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(54) Title: CHALCONE SYNTHASE DIHYDROFLAVONOL 4-REDUCTASE AND LEUCOANTHOCYANIDINE REDUCTASE FROM CLOVER, MEDIC RYEGRASS OR FESCUE

(57) Abstract: The present invention relates to nucleic acid fragments encoding amino acid sequences for flavonoid biosynthetic enzymes in plants, and the use thereof for the modification of, for example, flavonoid biosynthesis in plants, and more specifically the modification of the content of condensed tannins. In particularly preferred embodiments, the invention relates to the combinatorial expression of chalcone synthase (CHS) and/or dihydroflavonol 4-reductase (BAN) and/or leucoanthocyanidine reductase (LAR) in plants to modify, for example, flavonoid biosynthesis or more specifically the content of condensed tannins.

Chalcone Synthase, Dihydroflavonol 4-reductase and Leucoanthocyanidine reductase from Clover, Medic, Ryegrass or Fescue.

The present invention relates to nucleic acid fragments encoding amino acid sequences for flavonoid biosynthetic enzyme polypeptides in plants, and the use thereof for the modification of, for example, flavonoid biosynthesis in plants, and more specifically the modification of the content of condensed tannins. In particularly preferred embodiments, the invention relates to the combinatorial expression of chalcone synthase (CHS) and/or dihydroflavonol 4-reductase (BAN) and/or leucoanthocyanidine reductase (LAR) in plants to modify, for example, flavonoid biosynthesis or more specifically the content of condensed tannins.

Flavonoids constitute a relatively diverse family of aromatic molecules that are derived from phenylalanine and malonyl-coenzyme A (CoA, via the fatty acid pathway). These compounds include six major subgroups that are found in most higher plants: the chalcones, flavones, flavonols, flavandiols, anthocyanins and condensed tannins (or proanthocyanidins). A seventh group, the aurones, is widespread, but not ubiquitous.

Some plant species also synthesize specialised forms of flavonoids, such as the isoflavonoids that are found in legumes and a small number of non-legume plants. Similarly, sorghum, maize and gloxinia are among the few species known to synthesize 3-deoxyanthocyanins (or phlobaphenes in the polymerised form). The stilbenes, which are closely related to flavonoids, are synthesised by another group of unrelated species that includes grape, peanut and pine.

Besides providing pigmentation to flowers, fruits, seeds, and leaves, flavonoids also have key roles in signalling between plants and microbes, in male fertility of some species, in defence as antimicrobial agents and feeding deterrents, and in UV protection.

Flavonoids also have significant activities when ingested by animals, and there is great interest in their potential health benefits, particularly for compounds such as isoflavonoids, which have been linked to anticancer benefits, and stilbenes that are believed to contribute to reduced heart disease. Condensed tannins which are plant polyphenols with protein-precipitating and antioxidant properties are involved in protein binding, metal chelation, anti-oxidation, and UV-light absorption. As a result condensed tannins inhibit viruses, microorganisms, insects, fungal pathogens, and monogastric digestion. Moderate amounts of

tannins improve forage quality by disrupting protein foam and conferring protection from rumen pasture bloat. Bloat is a digestive disorder that occurs on some highly nutritious forage legumes such as alfalfa (*Medicago sativa*) and white clover (*Trifolium repens*). Moderate amounts of tannin can also reduce digestion rates in the rumen and can reduce parasitic load sufficiently to increase the titre of amino acids and small peptides in the small intestine without compromising total digestion.

The major branch pathways of flavonoid biosynthesis start with general phenylpropanoid metabolism and lead to the nine major subgroups: the colourless chalcones, aurones, isoflavonoids, flavones, flavonols, flavandiols, anthocyanins, condensed tannins, and phlobaphene pigments. The enzyme phenylalanine ammonia-lyase (PAL) of the general phenylpropanoid pathway will lead to the production of cinnamic acid. Cinnamate-4-hydroxylase (C4H) will produce p-coumaric acid which will be converted through the action of 4-coumaroyl:CoA-ligase (4CL) to the production of 4-coumaroyl-CoA and malonyl-CoA. The first committed step channelling carbon into the flavonoid biosynthesis pathway is catalysed by chalcone synthase (CHS), which uses malonyl CoA and 4-coumaryl CoA as substrates.

The *Arabidopsis BANYULS* gene encodes a dihydroflavonol 4-reductase-like protein (BAN) that may be an anthocyanine reductase (ACR). The reaction catalysed by BAN is considered to be one possible branching point from the general flavonoid pathway to the condensed tannin biosynthesis.

An alternative pathway to condensed tannins is via leucoanthocyanidine reductase (LAR). LAR utilises the same substrate as the ACR (BAN) but produces a 2,3-trans isomer as compared to the 2,3-cis isomer produced by ACR.

While nucleic acid sequences encoding the key enzymes in the condensed tannins biosynthetic pathway CHS, BAN and LAR have been isolated for certain species of plants, there remains a need for materials useful in modifying flavonoid biosynthesis and more specifically in modifying condensed tannin biosynthesis and therewith in modifying forage quality, for example by disrupting protein foam and conferring protection from rumen pasture bloat, particularly in forage legumes and grasses, including alfalfa, medics, clovers, ryegrasses and fescues, and for methods for their use.

It is an object of the present invention to overcome, or at least alleviate, one or more of the difficulties or deficiencies associated with the prior art.

In one aspect, the present invention provides substantially purified or isolated nucleic acids or nucleic acid fragments encoding key polypeptide enzymes in the condensed tannins biosynthetic pathway CHS, BAN and LAR, or functionally active fragments or variants of these enzymes, from a clover (*Trifolium*), medic (*Medicago*), ryegrass (*Lolium*) or fescue (*Festuca*) species.

The present invention also provides substantially purified or isolated nucleic acids or nucleic acid fragments encoding amino acid sequences for a class of polypeptides which are related to CHS, BAN and LAR or functionally active fragments or variants of CHS, BAN or LAR. Such polypeptides are referred to herein as CHS-like, BAN-like and LAR-like, respectively, and includes polypeptides having similar functional activity.

The individual or simultaneous enhancement or otherwise manipulation of CHS, BAN and LAR or like gene activities in plants may enhance or otherwise alter flavonoid biosynthesis; may enhance or otherwise alter the plant capacity for protein binding, metal chelation, anti-oxidation, and UV-light absorption; may enhance or reduce or otherwise alter plant pigment production; and may enhance or otherwise alter the amount of condensed tannins contained within forage legumes and grasses, including alfalfa, medics, clovers, ryegrasses and fescues and therewith the capacity to reduce bloating by disrupting protein foam.

Methods for the manipulation of CHS, BAN and LAR or like gene activities in plants, including legumes such as clovers (*Trifolium* species), lucerne (*Medicago sativa*) and grass species such as ryegrasses (*Lolium* species) and fescues (*Festuca* species) may facilitate the production of, for example, forage legumes and forage grasses and other crops with enhanced tolerance to biotic stresses such as viruses, microorganisms, insects and fungal pathogens; altered pigmentation in flowers; forage legumes with enhanced herbage quality and bloat-safety.

The clover (*Trifolium*), medic (*Medicago*), ryegrass (*Lolium*) or fescue (*Festuca*) species may be of any suitable type, including white clover (*Trifolium repens*), red clover (*Trifolium pratense*), subterranean clover (*Trifolium*

subterraneum), alfalfa (*Medicago sativa*), Italian or annual ryegrass (*Lolium multiflorum*), perennial ryegrass (*Lolium perenne*), tall fescue (*Festuca arundinacea*), meadow fescue (*Festuca pratensis*) and red fescue (*Festuca rubra*). Preferably the species is a clover or a ryegrass, more preferably white clover (*T. repens*) or perennial ryegrass (*L. perenne*). White clover (*Trifolium repens* L.) and perennial ryegrass (*Lolium perenne* L.) are key pasture legumes and grasses, respectively, in temperate climates throughout the world. Perennial ryegrass is also an important turf grass.

The nucleic acid or nucleic acid fragment may be of any suitable type and includes DNA (such as cDNA or genomic DNA) and RNA (such as mRNA) that is single- or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases, and combinations thereof. The RNA is readily obtainable, for example, by transcription of a DNA sequence according to the present invention, to produce an RNA corresponding to the DNA sequence. The RNA may be synthesised, *in vivo* or *in vitro* or by chemical synthesis to produce a sequence corresponding to a DNA sequence by methods well known in the art. In this specification, where the degree of sequence similarity between an RNA and DNA is such that the strand of the DNA could encode the RNA, then the RNA is said to "correspond" to that DNA.

In a preferred embodiment of this aspect of the invention, the substantially purified or isolated nucleic acid or nucleic acid fragment encoding a CHS or CHS-like protein includes the nucleotide sequences shown in Figures 2, 6, 10 and 14 hereto (Sequence ID Nos. 1, 3, 5 and 7, respectively); (b) complements of the sequences recited in (a); (c) sequences antisense to the sequences recited in (a) and (b); and (d) functionally active fragments and variants of the sequences recited in (a), (b) and (c); and (e) RNA sequences corresponding to the sequences recited in (a), (b), (c), and (d).

In a further preferred embodiment of this aspect of the invention, the substantially purified or isolated nucleic acid or nucleic acid fragment encoding a BAN or BAN-like protein includes the nucleotide sequence shown in Figure 18 hereto (Sequence ID No. 9); (b) complements of the sequence recited in (a); (c) sequences antisense to the sequences recited in (a) and (b); and (d) functionally

active fragments and variants of the sequences recited in (a), (b) and (c); and (e) RNA sequences corresponding to the sequences recited in (a), (b), (c), and (d).

In a still further preferred embodiment of this aspect of the invention, the substantially purified or isolated nucleic acid or nucleic acid fragment encoding a LAR or LAR-like protein includes the nucleotide sequence shown in Figures 22, 26 and 30 hereto (Sequence ID Nos. 11, 13 and 15 respectively); (b) complements of the sequences recited in (a); (c) sequences antisense to the sequences recited in (a) and (b); and (d) functionally active fragments and variants of the sequences recited in (a), (b) and (c); and (e) RNA sequences corresponding to the sequences recited in (a), (b), (c), and (d).

The term "isolated" means that the material is removed from its original environment (e.g. the natural environment if it is naturally occurring). For example, a naturally occurring nucleic acid or polypeptide present in a living plant is not isolated, but the same nucleic acid or polypeptide separated from some or all of the coexisting materials in the natural system, is isolated. Such nucleic acids could be part of a vector and/or such nucleic acids could be part of a composition, and still be isolated in that such a vector or composition is not part of its natural environment. An isolated polypeptide could be part of a composition and still be isolated in that such a composition is not part of its natural environment.

The term "purified" means that the nucleic acid or polypeptide is substantially free of other nucleic acids or polypeptides.

By "functionally active" in respect of a nucleic acid it is meant that the fragment or variant (such as an analogue, derivative or mutant) is capable of modifying flavonoid biosynthesis in a plant. Such variants include naturally occurring allelic variants and non-naturally occurring variants. Additions, deletions, substitutions and derivatizations of one or more of the nucleotides are contemplated so long as the modifications do not result in loss of functional activity of the fragment or variant. Preferably the functionally active fragment or variant has at least approximately 80% identity to the relevant part of the above mentioned sequence, more preferably at least approximately 90% identity, most preferably at least approximately 95% identity. Such functionally active variants and fragments include, for example, those having nucleic acid changes which result in conservative amino acid substitutions of one or more residues in the

corresponding amino acid sequence. Preferably the fragment has a size of at least 30 nucleotides, more preferably at least 45 nucleotides, most preferably at least 60 nucleotides.

By "functionally active" in respect of a polypeptide is meant that the fragment or variant has one or more of the biological properties or functions of the polypeptides CHS, CHS-like, BAN, BAN-like, LAR and LAR-like, respectively. Additions, deletions, substitutions and derivatizations of one or more of the amino acids are contemplated so long as the modifications do not result in loss of functional activity of the fragment or variant. Preferably the functionally active fragment or variant has at least approximately 60% identity to the relevant part of the above mentioned sequence, more preferably at least approximately 80% identity, most preferably at least approximately 90% identity. Such functionally active variants and fragments include, for example, those having conservative amino acid substitutions of one or more residues in the corresponding amino acid sequence. Preferably the fragment has a size of at least 10 amino acids, more preferably at least 15 amino acids, most preferably at least 20 amino acids.

The term "construct" as used herein refers to an artificially assembled or isolated nucleic acid molecule which includes the gene of interest. In general a construct may include the gene or genes of interest, a marker gene which in some cases can also be the gene of interest and appropriate regulatory sequences. It should be appreciated that the inclusion of regulatory sequences in a construct is optional, for example, such sequences may not be required in situations where the regulatory sequences of a host cell are to be used. The term construct includes vectors but should not be seen as being limited thereto.

The term "vector" as used herein encompasses both cloning and expression vectors. Vectors are often recombinant molecules containing nucleic acid molecules from several sources.

By "operatively linked" is meant that said regulatory element(s) is capable of causing expression of said nucleic acid(s) or nucleic acid fragment(s) in a plant cell and said terminator(s) is capable of terminating expression of said nucleic acid(s) or nucleic acid fragment(s) in a plant cell. Preferably, said regulatory element(s) is upstream of said nucleic acid(s) or nucleic acid fragment(s) and said terminator(s) is downstream of said nucleic acid(s) or nucleic acid fragment(s). In

a particularly preferred embodiment, each nucleic acid or nucleic acid fragment has one or more upstream promoters and one or more downstream terminators, although expression of more than one nucleic acid or nucleic acid fragment from an upstream regulatory element(s) or termination of more than one nucleic acid or nucleic acid fragment from a downstream terminator(s) is not precluded.

By "an effective amount" it is meant an amount sufficient to result in an identifiable phenotypic trait in said plant, or a plant, plant seed or other plant part derived therefrom. Such amounts can be readily determined by an appropriately skilled person, taking into account the type of plant, the route of administration and other relevant factors. Such a person will readily be able to determine a suitable amount and method of administration. See, for example, Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, the entire disclosure of which is incorporated herein by reference.

It will also be understood that the term "comprises" (or its grammatical variants) as used in this specification is equivalent to the term "includes" and should not be taken as excluding the presence of other elements or features.

Genes encoding other CHS or CHS-like, BAN or BAN-like and LAR or LAR-like proteins, either as cDNAs or genomic DNAs, may be isolated directly by using all or a portion of the nucleic acids or nucleic acid fragments of the present invention as hybridisation probes to screen libraries from the desired plant employing the methodology well known to those skilled in the art. Specific oligonucleotide probes based upon the nucleic acid sequences of the present invention may be designed and synthesized by methods known in the art. Moreover, the entire sequences may be used directly to synthesize DNA probes by methods known to the skilled artisan such as random primer DNA labelling, nick translation, or end-labelling techniques, or RNA probes using available *in vitro* transcription systems. In addition, specific primers may be designed and used to amplify a part or all of the sequences of the present invention. The resulting amplification products may be labelled directly during amplification reactions or labelled after amplification reactions, and used as probes to isolate full-length cDNA or genomic fragments under conditions of appropriate stringency.

In addition, short segments of the nucleic acids or nucleic acid fragments of the present invention may be used in protocols to amplify longer nucleic acids or

nucleic acid fragments encoding homologous genes from DNA or RNA. For example, polymerase chain reaction may be performed on a library of cloned nucleic acid fragments wherein the sequence of one primer is derived from the nucleic acid sequences of the present invention, and the sequence of the other primer takes advantage of the presence of the polyadenylic acid tracts to the 3' end of the mRNA precursor encoding plant genes. Alternatively, the second primer sequence may be based upon sequences derived from the cloning vector. For example, those skilled in the art can follow the RACE protocol [Frohman *et al.* (1988), *Proc. Natl. Acad. Sci. USA* 85:8998, the entire disclosure of which is incorporated herein by reference] to generate cDNAs by using PCR to amplify copies of the region between a single point in the transcript and the 3' or 5' end. Using commercially available 3' RACE and 5' RACE systems (BRL), specific 3' or 5' cDNA fragments may be isolated [Ohara *et al.* (1989), *Proc. Natl. Acad. Sci. USA* 86:5673; Loh *et al.* (1989), *Science* 243:217, the entire disclosures of which are incorporated herein by reference]. Products generated by the 3' and 5' RACE procedures may be combined to generate full-length cDNAs.

In a second aspect of the present invention there is provided a substantially purified or isolated polypeptide from a clover, (*Trifolium*), medic (*Medicago*), ryegrass (*Lolium*) or fescue (*Festuca*) species, selected from the group consisting of CHS and CHS-like, BAN and BAN-like, and LAR and LAR-like proteins; and functionally active fragments and variants thereof.

The clover (*Trifolium*), medic (*Medicago*), ryegrass (*Lolium*) or fescue (*Festuca*) species may be of any suitable type, including white clover (*Trifolium repens*), red clover (*Trifolium pratense*), subterranean clover (*Trifolium subterraneum*), alfalfa (*Medicago sativa*), Italian or annual ryegrass (*Lolium multiflorum*), perennial ryegrass (*Lolium perenne*), tall fescue (*Festuca arundinacea*), meadow fescue (*Festuca pratensis*) and red fescue (*Festuca rubra*). Preferably the species is a clover or a ryegrass, more preferably white clover (*T. repens*) or perennial ryegrass (*L. perenne*).

In a preferred embodiment of this aspect of the invention, the substantially purified or isolated CHS or CHS-like polypeptide includes an amino acid sequence selected from the group consisting of sequences shown in Figures 3, 7, 11 and 15

hereto (Sequence ID Nos. 2, 4, 6 and 8, respectively) and functionally active fragments and variants thereof.

In a further preferred embodiment of this aspect of the invention, the substantially purified or isolated BAN or BAN-like polypeptide includes an amino acid sequence shown in Figure 19 hereto (Sequence ID No. 10), and functionally active fragments and variants thereof.

In a still further preferred embodiment of this aspect of the invention, the substantially purified or isolated LAR or LAR-like polypeptide includes an amino acid sequence selected from the group consisting of sequences shown in Figures 23, 27 and 31 hereto (Sequence ID Nos. 12, 14 and 16, respectively), and functionally active fragments and variants thereof.

In a further embodiment of this aspect of the invention, there is provided a polypeptide produced (e.g. recombinantly) from a nucleic acid or nucleic acid fragment according to the present invention. Techniques for recombinantly producing polypeptides are well known to those skilled in the art.

Availability of the nucleotide sequences of the present invention and deduced amino acid sequences facilitates immunological screening of cDNA expression libraries. Synthetic peptides representing portions of the instant amino acid sequences may be synthesized. These peptides may be used to immunise animals to produce polyclonal or monoclonal antibodies with specificity for peptides and/or proteins including the amino acid sequences. These antibodies may be then used to screen cDNA expression libraries to isolate full-length cDNA clones of interest.

In a still further aspect of the present invention there is provided a construct including one or more nucleic acids or nucleic acid fragments according to the present invention.

In a particularly preferred embodiment the construct may include nucleic acids or nucleic acid fragments encoding both CHS or CHS-like and BAN or BAN-like polypeptides.

In another preferred embodiment the construct may include nucleic acids or nucleic acid fragments encoding both CHS or CHS-like and LAR or LAR-like polypeptides.

In yet another preferred embodiment the construct may include nucleic acids or nucleic acid fragments encoding both LAR or LAR-like and BAN or BAN-like polypeptides.

In an even more preferred embodiment the construct may include nucleic acids or nucleic acid fragments encoding all three of CHS or CHS-like, BAN or BAN-like and LAR or LAR-like polypeptides.

Constructs including nucleic acids or nucleic acid fragments encoding CHS or CHS-like and BAN or BAN-like, and optionally further including nucleic acids or nucleic acid fragments encoding LAR or LAR-like, are particularly preferred.

In a still further aspect of the present invention there is provided a vector including one or more nucleic acids or nucleic acid fragments according to the present invention.

In a preferred embodiment of this aspect of the invention, the construct may include one or several of the following: one or more regulatory elements such as promoters, one or more nucleic acids or nucleic acid fragments according to the present invention and one or more terminators; said one or more regulatory elements, one or more nucleic acids or nucleic acid fragments and one or more terminators being operatively linked.

In a particularly preferred embodiment the construct may contain nucleic acids or nucleic acid fragments encoding both CHS or CHS-like and BAN or BAN-like polypeptides, operatively linked to a regulatory element or regulatory elements, such that both CHS or CHS-like and BAN or BAN-like polypeptides are expressed.

In another preferred embodiment the construct may contain nucleic acids or nucleic acid fragments encoding both CHS or CHS-like and LAR or LAR-like polypeptides, operatively linked to a regulatory element or regulatory elements, such that both CHS or CHS-like and LAR or LAR-like polypeptides are expressed.

In yet another preferred embodiment the construct may contain nucleic acids or nucleic acid fragments encoding both LAR or LAR-like and BAN or BAN-like polypeptides, operatively linked to a regulatory element or regulatory elements, such that both LAR or LAR-like and BAN or BAN-like polypeptides are expressed.

In an even more preferred embodiment the construct may contain nucleic acids or nucleic acid fragments encoding all three of CHS or CHS-like, BAN or BAN-like and LAR or LAR-like polypeptides, operatively linked to a regulatory element or regulatory elements, such that all three of CHS or CHS-like, BAN or BAN-like and LAR or LAR-like polypeptides are expressed.

Constructs including nucleic acids or nucleic acid fragments encoding CHS or CHS-like and BAN or BAN-like, and optionally further including nucleic acids or nucleic acid fragments encoding LAR or LAR-like, are particularly preferred.

The construct or vector may be of any suitable type and may be viral or non-viral. The vector may be an expression vector. Such vectors include chromosomal, non-chromosomal and synthetic nucleic acid sequences, e.g. derivatives of plant viruses; bacterial plasmids; derivatives of the Ti plasmid from *Agrobacterium tumefaciens*, derivatives of the Ri plasmid from *Agrobacterium rhizogenes*; phage DNA; yeast artificial chromosomes; bacterial artificial chromosomes; binary bacterial artificial chromosomes; vectors derived from combinations of plasmids and phage DNA. However, any other vector may be used as long as it is replicable, integrative or viable in the plant cell.

The regulatory element and terminator may be of any suitable type and may be endogenous to the target plant cell or may be exogenous, provided that they are functional in the target plant cell.

Preferably the regulatory element is a promoter. A variety of promoters which may be employed in the vectors of the present invention are well known to those skilled in the art. Factors influencing the choice of promoter include the desired tissue specificity of the vector, and whether constitutive or inducible expression is desired and the nature of the plant cell to be transformed (e.g. monocotyledon or dicotyledon). Particularly suitable promoters include but are not limited to the constitutive Cauliflower Mosaic Virus 35S (CaMV 35S) promoter and derivatives thereof, the maize Ubiquitin promoter, the rice Actin promoter, and the tissue-specific Arabidopsis small subunit (ASSU) promoter.

A variety of terminators which may be employed in the vectors and constructs of the present invention are also well known to those skilled in the art. The terminator may be from the same gene as the promoter sequence or a

different gene. Particularly suitable terminators are polyadenylation signals, such as the CaMV 35S polyA and other terminators from the nopaline synthase (*nos*), the octopine synthase (*ocs*) and the rbcS genes.

The construct or vector, in addition to the regulatory element(s), the nucleic acid(s) or nucleic acid fragment(s) of the present invention and the terminator(s), may include further elements necessary for expression of the nucleic acid(s) or nucleic acid fragment(s), in different combinations, for example vector backbone, origin of replication (ori), multiple cloning sites, recognition sites for recombination events, spacer sequences, enhancers, introns (such as the maize Ubiquitin *Ubi* intron), antibiotic resistance genes and other selectable marker genes [such as the neomycin phosphotransferase (*npt2*) gene, the hygromycin phosphotransferase (*hph*) gene, the phosphinotricin acetyltransferase (*bar* or *pat*) gene and the gentamycin acetyl transferase (*aacC1*) gene], and reporter genes [such as beta-glucuronidase (GUS) gene (*gusA*) and green fluorescent protein (gfp)]. The vector may also contain a ribosome binding site for translation initiation. The vector may also include appropriate sequences for amplifying expression.

As an alternative to use of a selectable marker gene to provide a phenotypic trait for selection of transformed host cells, the presence of the vector in transformed cells may be determined by other techniques well known in the art, such as PCR (polymerase chain reaction), Southern blot hybridisation analysis, histochemical GUS assays, visual examination including microscopic examination of fluorescence emitted by gfp, northern and Western blot hybridisation analyses.

Those skilled in the art will appreciate that the various components of the construct or vector are operatively linked, so as to result in expression of said nucleic acid(s) or nucleic acid fragment(s). Techniques for operatively linking the components of the vector of the present invention are well known to those skilled in the art. Such techniques include the use of linkers, such as synthetic linkers, for example including one or more restriction enzyme sites.

The constructs and vectors of the present invention may be incorporated into a variety of plants, including monocotyledons (such as grasses from the genera *Lolium*, *Festuca*, *Paspalum*, *Pennisetum*, *Panicum* and other forage and turfgrasses, corn, oat, sugarcane, wheat and barley), dicotyledons (such as *Arabidopsis*, tobacco, clovers, medics, eucalyptus, potato, sugarbeet, canola,

soybean, chickpea) and gymnosperms. In a preferred embodiment, the vectors may be used to transform monocotyledons, preferably grass species such as ryegrasses (*Lolium* species) and fescues (*Festuca* species), more preferably perennial ryegrass, including forage- and turf-type cultivars. In an alternate preferred embodiment, the constructs and vectors may be used to transform dicotyledons, preferably forage legume species such as clovers (*Trifolium* species) and medics (*Medicago* species), more preferably white clover (*Trifolium repens*), red clover (*Trifolium pratense*), subterranean clover (*Trifolium subterraneum*) and alfalfa (*Medicago sativa*). Clovers, alfalfa and medics are key pasture legumes in temperate climates throughout the world.

Techniques for incorporating the constructs and vectors of the present invention into plant cells (for example by transduction, transfection or transformation) are known to those skilled in the art. Such techniques include *Agrobacterium*-mediated introduction, electroporation to tissues, cells and protoplasts, protoplast fusion, injection into reproductive organs, injection into immature embryos and high velocity projectile introduction to cells, tissues, calli, immature and mature embryos. The choice of technique will depend largely on the type of plant to be transformed.

In a further aspect of the present invention there is provided a method of isogenic transformation of a dicotyledonous plant, said method including transforming only one of each pair of cotyledons. This enables the production of pairs of transgenic plant and corresponding untransformed negative control in an otherwise isogenic genetic background for detailed functional assessment of the impact of the transgene on plant phenotype. In a preferred embodiment of this aspect of the invention, the method may include isogenic transformation of a dicotyledonous plant with a construct or vector according to the present invention.

Cells incorporating the constructs and vectors of the present invention may be selected, as described above, and then cultured in an appropriate medium to regenerate transformed plants, using techniques well known in the art. The culture conditions, such as temperature, pH and the like, will be apparent to the person skilled in the art. The resulting plants may be reproduced, either sexually or asexually, using methods well known in the art, to produce successive generations of transformed plants.

In a further aspect of the present invention there is provided a plant cell, plant, plant seed or other plant part, including, e.g. transformed with, one or more constructs, vectors, nucleic acids or nucleic acid fragments of the present invention.

The plant cell, plant, plant seed or other plant part may be from any suitable species, including monocotyledons, dicotyledons and gymnosperms. In a preferred embodiment the plant cell, plant, plant seed or other plant part may be from a monocotyledon, preferably a grass species, more preferably a ryegrass (*Lolium* species) or fescue (*Festuca* species), more preferably perennial ryegrass, including both forage- and turf-type cultivars. In an alternate preferred embodiment the plant cell, plant, plant seed or other plant part may be from a dicotyledon, preferably forage legume species such as clovers (*Trifolium* species) and medics (*Medicago* species), more preferably white clover (*Trifolium repens*), red clover (*Trifolium pratense*), subterranean clover (*Trifolium subterraneum*) and alfalfa (*Medicago sativa*).

The present invention also provides a plant, plant seed or other plant part, or a plant extract derived from a plant cell of the present invention.

The present invention also provides a plant, plant seed or other plant part, or a plant extract derived from a plant of the present invention.

In a further aspect of the present invention there is provided a method of modifying condensed tannin biosynthesis; of modifying flavonoid biosynthesis; of modifying protein binding, metal chelation, anti-oxidation, and UV-light absorption; of modifying plant pigment production; of modifying plant defence to biotic stresses such as viruses, microorganisms, insects, fungal pathogens; of modifying forage quality by disrupting protein foam and conferring protection from rumen pasture bloat, said method including introducing into said plant an effective amount of a nucleic acid or nucleic acid fragment, construct and/or vector according to the present invention.

In a particularly preferred embodiment the method may include introducing into said plant nucleic acids or nucleic acid fragments encoding both CHS or CHS-like and BAN or BAN-like polypeptides.

In another preferred embodiment the method may include introducing into said plant nucleic acids or nucleic acid fragments encoding both CHS or CHS-like and LAR or LAR-like polypeptides.

In yet another preferred embodiment the method may include introducing into said plant nucleic acids or nucleic acid fragments encoding both LAR or LAR-like and BAN or BAN-like polypeptides.

In an even more preferred embodiment the method may include introducing into said plant nucleic acids or nucleic acid fragments encoding all three of CHS or CHS-like, BAN or BAN-like and LAR or LAR-like polypeptides.

Methods including the combinatorial expression of nucleic acids or nucleic acid fragments encoding CHS or CHS-like and BAN or BAN-like, and optionally further including the use of nucleic acids or nucleic acid fragments encoding LAR or LAR-like, are particularly preferred.

In a further aspect of the present invention there is provided a method of inhibiting bloat in an animal, said method including providing the animal with a forage plant including a construct, vector, nucleic acid or nucleic acid fragment according to the present invention. The animal is preferably a ruminant, including sheep, goats and cattle. The forage plant including a construct vector, nucleic acid or nucleic acid fragment according to the present invention may form all or part of the feed of the animal. The forage plant preferably expresses CHS or CHS-like proteins, BAN or BAN-like proteins, and/or LAR or LAR-like proteins at higher levels than the equivalent wild-type plant. More preferably, the forage plant expresses both CHS or CHS-like proteins and BAN or BAN-like proteins; both CHS or CHS-like proteins and LAR or LAR-like proteins; or both BAN or BAN-like proteins and LAR or LAR-like proteins; at higher levels than the equivalent wild-type plant. More preferably, the forage plant expresses all three of CHS or CHS-like proteins, BAN or BAN-like proteins, and LAR or LAR-like proteins; at higher levels than the equivalent wild-type plant.

In a further aspect of the present invention there is provided a method for enhancing an animal's growth rate, said method including providing the animal with a forage plant including a construct, vector, nucleic acid or nucleic acid fragment according to the present invention. The animal is preferably a ruminant,

including sheep, goats and cattle. The forage plant including a construct, vector, nucleic acid or nucleic acid fragment according to the present invention may form all or part of the feed of the animal. The forage plant preferably expresses CHS or CHS-like proteins, BAN or BAN-like proteins, and/or LAR or LAR-like proteins at higher levels than the equivalent wild-type plant. More preferably, the forage plant expresses both CHS or CHS-like proteins and BAN or BAN-like proteins; both CHS or CHS-like proteins and LAR or LAR-like proteins; or both BAN or BAN-like proteins and LAR or LAR-like proteins; at higher levels than the equivalent wild-type plant. More preferably, the forage plant expresses all three of CHS or CHS-like proteins, BAN or BAN-like proteins, and LAR or LAR-like proteins; at higher levels than the equivalent wild-type plant.

It is estimated that the method of enhancing an animal's growth rate according to this invention should result in an increase in, for example, lamb growth rate of at least approximately 5%, more preferably at least approximately 10%.

Using the methods and materials of the present invention, condensed tannin biosynthesis, flavonoid biosynthesis, protein binding, metal chelation, anti-oxidation, UV-light absorption, tolerance to biotic stresses such as viruses, microorganisms, insects and fungal pathogens; pigmentation in for example flowers and leaves; herbage quality and bloat-safety; isoflavonoid content leading to health benefits, may be increased or otherwise altered, for example by incorporating additional copies of one or more sense nucleic acids or nucleic acid fragments of the present invention. They may be decreased or otherwise altered, for example by incorporating one or more antisense nucleic acids or nucleic acid fragments of the present invention.

Documents cited in this specification are for reference purposes only and their inclusion is not acknowledgment that they form part of the common general knowledge in the relevant art.

The present invention will now be more fully described with reference to the accompanying Examples and drawings. It should be understood, however, that the description following is illustrative only and should not be taken in any way as a restriction on the generality of the invention described above.

In the Figures

Figure 1 shows the plasmid map in pGEM-T Easy of TrCHSa3.

Figure 2 shows the nucleotide sequence of TrCHSa3 (Sequence ID No. 1).

Figure 3 shows the deduced amino acid sequence of TrCHSa3 (Sequence ID No. 2).

Figure 4 shows plasmid maps of sense and antisense constructs of TrCHSa3 in the binary vector pPZP221:35S^2.

Figure 5 shows the plasmid map in pGEM-T Easy of TrCHSc.

Figure 6 shows the nucleotide sequence of TrCHSc (Sequence ID No. 3).

Figure 7 shows the deduced amino acid sequence of TrCHSc (Sequence ID No. 4).

Figure 8 shows plasmid maps of sense and antisense constructs of TrCHSc in the binary vector pPZP221:35S^2.

Figure 9 shows the plasmid map in pGEM-T Easy of TrCHSf.

Figure 10 shows the nucleotide sequence of TrCHSf (Sequence ID No. 5).

Figure 11 shows the deduced amino acid sequence of TrCHSf (Sequence ID No. 6).

Figure 12 shows plasmid maps of sense and antisense constructs of TrCHSf in the binary vector pPZP221:35S^2.

Figure 13 shows the plasmid map in pGEM-T Easy of TrCHSh.

Figure 14 shows the nucleotide sequence of TrCHSh (Sequence ID No. 7).

Figure 15 shows the deduced amino acid sequence of TrCHSh (Sequence ID No. 8).

Figure 16 shows plasmid maps of sense and antisense constructs of TrCHSh in the binary vector pPZP221:35S^2.

Figure 17 shows the plasmid map in pGEM-T Easy of TrBANa.

Figure 18 shows the nucleotide sequence of TrBANa (Sequence ID No. 9).

Figure 19 shows the deduced amino acid sequence of TrBANa (Sequence ID No. 10).

Figure 20 shows plasmid maps of sense and antisense constructs TrBANa in the binary vector pPZP221:35S^2.

Figure 21 shows the plasmid map in pGEM-T Easy of TrLARa.

Figure 22 shows the nucleotide sequence of TrLARa (Sequence ID No. 11).

Figure 23 shows the deduced amino acid sequence of TrLARa (Sequence ID No. 12).

Figure 24 shows plasmid maps of sense and antisense constructs of TrLARa in the binary vector pPZP221:35S^2.

Figure 25 shows the plasmid map in pGEM-T Easy of TrLARb.

Figure 26 shows the nucleotide sequence of TrLARb (Sequence ID No. 13).

Figure 27 shows the deduced amino acid sequence of TrLARb (Sequence ID No. 14).

Figure 28 shows plasmid maps of sense and antisense constructs of TrLARb in the binary vector pPZP221:35S^2.

Figure 29 shows the plasmid map in pGEM-T Easy of TrLARc.

Figure 30 shows the nucleotide sequence of TrLARc (Sequence ID No. 15).

Figure 31 shows the deduced amino acid sequence of TrLARc (Sequence ID No. 16).

Figure 32 shows plasmid maps of sense and antisense constructs of TrLARc in the binary vector pPZP221:35S^2.

Figure 33 shows the plasmid map of the binary vector pPZP221:ASSU::TrBAN:35S^2::TrCHS.

Figure 34 shows the plasmid maps of the modular vector system comprising a binary base vector and 7 auxiliary vectors.

Figure 35 shows an example of the modular binary transformation vector system comprising plasmid maps of the binary transformation vector backbone and 4

expression cassettes in auxiliary vectors (A) and the plasmid map of the T-DNA region of the final binary transformation vector.

Figure 36 shows A, white clover cotyledons; B, C, D, selection of plantlets transformed with a binary transformation vector constructed as described in Examples 4 and 5; E, putative transgenic white clover on root-inducing medium; F, G, white clover plants transgenic for genes involved in condensed tannin biosynthesis.

Figure 37 shows the molecular analysis of white clover plants transgenic for the TrBAN gene with Q-PCR amplification plot, agarose gel of PCR product and Southern hybridisation blot.

Figure 38 shows the molecular analysis of white clover plants transgenic for the TrCHSf gene with Q-PCR amplification plot and agarose gel of PCR product.

Figure 39 shows the molecular analysis of white clover plants transgenic for the TrLARb gene with Q-PCR amplification plot, agarose gel of PCR product and Southern hybridisation blot.

EXAMPLE 1

Preparation of cDNA libraries, isolation and sequencing of cDNAs coding for CHS, CHS-like, BAN, BAN-like, LAR and LAR-like proteins from white clover (*Trifolium repens*)

cDNA libraries representing mRNAs from various organs and tissues of white clover (*Trifolium repens*) were prepared. The characteristics of the white clover libraries are described below (Table 1).

TABLE 1

cDNA libraries from white clover (*Trifolium repens*)

Library	Organ/Tissue
01wc	Whole seedling, light grown
02wc	Nodulated root 3, 5, 10, 14, 21 &28 day old seedling
03wc	Nodules pinched off roots of 42 day old rhizobium inoculated plants
04wc	Cut leaf and stem collected after 0, 1, 4, 6 &14 h after cutting
05wc	Inflorescences: <50% open, not fully open and fully open

Library	Organ/Tissue
06wc	Dark grown etiolated
07wc	Inflorescence – very early stages, stem elongation, < 15 petals, 15-20 petals
08wc	seed frozen at –80°C, imbibed in dark overnight at 10°C
09wc	Drought stressed plants
10wc	AMV infected leaf
11wc	WCMV infected leaf
12wc	Phosphorus starved plants
13wc	Vegetative stolon tip
14wc	stolon root initials
15wc	Senescing stolon
16wc	Senescing leaf

The cDNA libraries may be prepared by any of many methods available. For example, total RNA may be isolated using the Trizol method (Gibco-BRL, USA) or the RNeasy Plant Mini kit (Qiagen, Germany), following the manufacturers' instructions. cDNAs may be generated using the SMART PCR cDNA synthesis kit (Clontech, USA), cDNAs may be amplified by long distance polymerase chain reaction using the Advantage 2 PCR Enzyme system (Clontech, USA), cDNAs may be cleaned using the GeneClean spin column (Bio 101, USA), tailed and size fractionated, according to the protocol provided by Clontech. The cDNAs may be introduced into the pGEM-T Easy Vector system 1 (Promega, USA) according to the protocol provided by Promega. The cDNAs in the pGEM-T Easy plasmid vector are transfected into *Escherichia coli* Epicurean coli XL10-Gold ultra competent cells (Stratagene, USA) according to the protocol provided by Stratagene.

Alternatively, the cDNAs may be introduced into plasmid vectors for first preparing the cDNA libraries in Uni-ZAP XR vectors according to the

manufacturer's protocol (Stratagene Cloning Systems, La Jolla, CA, USA). The Uni-ZAP XR libraries are converted into plasmid libraries according to the protocol provided by Stratagene. Upon conversion, cDNA inserts will be contained in the plasmid vector pBlueScript. In addition, the cDNAs may be introduced directly into precut pBlueScript II SK(+) vectors (Stratagene) using T4 DNA ligase (New England Biolabs), followed by transfection into *E. coli* DH10B cells according to the manufacturer's protocol (GIBCO BRL Products).

Once the cDNA inserts are in plasmid vectors, plasmid DNAs are prepared from randomly picked bacterial colonies containing recombinant plasmids, or the insert cDNA sequences are amplified via polymerase chain reaction using primers specific for vector sequences flanking the inserted cDNA sequences. Plasmid DNA preparation may be performed robotically using the Qiagen QiaPrep Turbo kit (Qiagen, Germany) according to the protocol provided by Qiagen. Amplified insert DNAs are sequenced in dye-terminator sequencing reactions to generate partial cDNA sequences (expressed sequence tags or "ESTs"). The resulting ESTs are analysed using an Applied Biosystems ABI 3700 sequence analyser.

EXAMPLE 2

DNA sequence analyses

The cDNA clones encoding CHS, CHS-like, BAN, BAN-like, LAR and LAR-like proteins were identified by conducting BLAST (Basic Local Alignment Search Tool; Altschul *et al.* (1993), *J. Mol. Biol.* 215:403-410) searches. The cDNA sequences obtained were analysed for similarity to all publicly available DNA sequences contained in the eBioinformatics nucleotide database using the BLASTN algorithm provided by the National Center for Biotechnology Information (NCBI). The DNA sequences were translated in all reading frames and compared for similarity to all publicly available protein sequences contained in the SWISS-PROT protein sequence database using BLASTx algorithm (v 2.0.1) (Gish and States (1993), *Nature Genetics* 3:266-272) provided by the NCBI.

The cDNA sequences obtained and identified were then used to identify additional identical and/or overlapping cDNA sequences generated using the BLASTN algorithm. The identical and/or overlapping sequences were subjected to a multiple alignment using the CLUSTALw algorithm, and to generate a consensus contig sequence derived from this multiple sequence alignment. The

consensus contig sequence was then used as a query for a search against the SWISS-PROT protein sequence database using the BLASTx algorithm to confirm the initial identification.

EXAMPLE 3

Identification and full-length sequencing of cDNAs encoding white clover CHS, BAN and LAR proteins

To fully characterise for the purposes of the generation of probes for hybridisation experiments and the generation of transformation vectors, a set of cDNAs encoding white clover CHS, BAN and LAR proteins was identified and fully sequenced.

Full-length cDNAs were identified from our EST sequence database using relevant published sequences (NCBI databank) as queries for BLAST searches. Full-length cDNAs were identified by alignment of the query and hit sequences using Sequencher (Gene Codes Corp., Ann Arbor, MI 48108, USA). The original plasmid was then used to transform chemically competent XL-1 cells (prepared in-house, $CaCl_2$ protocol). After colony PCR (using HotStarTaq, Qiagen) a minimum of three PCR-positive colonies per transformation were picked for initial sequencing with M13F and M13R primers. The resulting sequences were aligned with the original EST sequence using Sequencher to confirm identity and one of the three clones was picked for full-length sequencing, usually the one with the best initial sequencing result.

Sequencing of TrBAN could be completed with M13F and M13R primers. Sequencing of TrCHSa3, TrCHSc, TrCHSf, TrCHSh, TrLARa, TrLARb and TrLARc was completed by primer walking, i.e. oligonucleotide primers were designed to the initial sequence and used for further sequencing. The sequences of the oligonucleotide primers are shown in Table 2.

Contigs were then assembled in Sequencher. The contigs include the sequences of the SMART primers used to generate the initial cDNA library as well as pGEM-T Easy vector sequence up to the EcoRI cut site both at the 5' and 3' end.

Plasmid maps and the full cDNA sequences of TrCHSa3, TrCHSc, TrCHSf, TrCHSh, TrBANa, TrLARa, TrLARb and TrLARc proteins were obtained (Figures 1, 2, 5, 6, 9, 10, 13, 14, 17, 18, 21, 22, 25, 26, 29 and 30).

TABLE 2

List of primers used for sequencing of the full-length cDNAs of TrCHSa3, TrCHSc, TrCHSf, TrCHSh, TrLARa, TrLARb and TrLARc

gene name	clone ID	sequencing primer	primer sequence (5'>3')
TrCHSa3	05wc1RsB06	05wc1RsB06.f1	AGGAGGCTGCAGTCAAGG
		05wc1RsB06.f2	TGCCTGAAATTGAGAAACC
		05wc1RsB06.f3	AAAGCTAGCCTTGAAGCC
TrCHSc	07wc1TsE12	07wc1TsE12.f1	TCGGACATAACTCATGTGG
		07wc1TsE12.f2	TTGGGTTGGAGAATAAGG
		07wc1TsE12.r1	TGGACATTTATTGGTTGC
		07wc1TsE12.r2	TATCATGTCTGGAAATGC
TrCHSf	07wc1UsD07	07wc1UsD07.f1	AGATTGCATCAAAGAATGG
		07wc1UsD07.r1	GGTCCAAAAGCCAATCC
TrCHSh	13wc2IsG04	13wc2IsG04.f1	TAAGACGAGACATAGTGG
		13wc2IsG04.r1	TATTCACTAAGCACATGC
TrLARa	05wc1CsA02	05wc1CsA02.f1	TCATTTCTGCAATAGGAGG
		05wc1CsA02.r1	ATCCACCTCAGGTGAACC
TrLARb	05wc3EsA03	05wc3EsA03.f1	AATAGGAGGCTCTGATGG
		05wc3EsA03r1	ATCCACCTCAGGTGAACC
TrLARc	07wc1VsF06	07wc1VsF06.f1	AGGCTCTGATGGCTTGC
		07wc1VsF06.r1	ATCCACCTCAGGTGAACC

EXAMPLE 4

Development of binary transformation vectors containing chimeric genes with cDNA sequences from white clover TrCHSa3, TrCHSc, TrCHSf, TrCHSh, TrBANa, TrLARa, TrLARb and TrLARc

To alter the expression of the proteins involved in flavonoid biosynthesis, and more specifically condensed tannin biosynthesis to improve herbage quality

and bloat-safety, a set of sense and antisense binary transformation vectors was produced.

cDNA fragments were generated by high fidelity PCR with a proofreading DNA polymerase using the original pGEM-T Easy plasmid cDNA as a template. The primers used (Table 3) contained recognition sites for appropriate restriction enzymes, for example EcoRI and XbaI, for directional and non-directional cloning into the target vector. After PCR amplification and restriction digest with the appropriate restriction enzyme (usually XbaI), the cDNA fragments were cloned into the corresponding site in a modified pPZP binary vector (Hajdukiewicz *et al.*, 1994). The pPZP221 vector was modified to contain the $35S^2$ cassette from pKYLX71:$35S^2$ (Schardl *et al.*, 1987) as follows: pKYLX71:$35S^2$ was cut with ClaI. The 5' overhang was filled in using Klenow and the blunt end was A-tailed with Taq polymerase. After cutting with EcoRI, the 2kb fragment with an EcoRI-compatible and a 3'-A tail was gel-purified. pPZP221 was cut with HindIII and the resulting 5' overhang filled in and T-tailed with Taq polymerase. The remainder of the original pPZP221 multi-cloning site was removed by digestion with EcoRI, and the expression cassette cloned into the EcoRI site and the 3' T overhang restoring the HindIII site. This binary vector contains between the left and right border the plant selectable marker gene aacC1 under the control of the 35S promoter and 35S terminator and the pKYLX71:$35S^2$-derived expression cassette with a CaMV 35S promoter with a duplicated enhancer region and an rbcS terminator.

Alternatively, the primers for the amplification of cDNA fragments contained *att*B sequences for use with recombinases utilising the GATEWAY® system (Invitrogen). The resulting PCR fragments were used in a recombination reaction with pDONR® vector (Invitrogen) to generate entry vectors. A GATEWAY® cloning cassette (Invitrogen) was introduced into the multicloning site of the pPZP221:$35S^2$ vector following the manufacturer's protocol. In a further recombination reaction, the cDNAs encoding the open reading frame sequences were transferred from the entry vector to the GATEWAY®-enabled pPZP221:$35S^2$ vector.

The orientation of the constructs (sense or antisense) was checked by restriction enzyme digest and sequencing which also confirmed the correctness of the sequence. Transformation vectors containing chimeric genes using full-length

open reading frame cDNAs encoding white clover TrCHSa3, TrCHSc, TrCHSf, TrCHSh, TrBANa, TrLARa, TrLARb and TrLARc proteins in sense and antisense orientation under the control of the CaMV 35S[2] promoter were generated (Figures 4, 8, 12, 16, 20, 24, 28 and 32).

TABLE 3

List of primers used to PCR-amplify the open reading frames

gene name	primer	primer sequence (5'->3')
TrCHSa3	05wc1RsB06f	GAATTCTAGAAGATATGGTGAGTGTAGCTG
	05wc1RsB06r	GAATTCTAGAATCACACATCTTATATAGCC
TrCHSa3	05wc1RsB06fG	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCTAGAAGATATGGTGAGTGTAGCTG
	05wc1RsB06rG	GGGGACCACTTTGTACAAGAAAGCTGGGTTCTAGAATCACACATCTTATATAGCC
TrCHSc	07wc1TsE12f	GAATTCTAGAAGAAGAAATATGGGAGACGAAGG
	07wc1TsE12r	GAATTCTAGAAAGACTTCATGCACACAAGTTCC
TrCHSf	07wc1UsD07f	GAATTCTAGATGATTCATTGTTTGTTTCCATAAC
	07wc1UsD07r	GAATTCTAGAACATATTCATCTTCCTATCAC
TrCHSh	13wc2IsG04f	GAATTCTAGATCCAAATTCTCGTACCTCACC
	13wc2IsG04r	GAATTCTAGATAGTTCACATCTCTCGGCAGG
TrBANa	05wc2XsG02f	GGATCCTCTAGAGCACTAGTGTGTATAAGTTTCTTGG
	05wc2XsG02r	GGATCCTCTAGACCCCCTTAGTCTTAAAATACTCG
TrLARa	05wc1CsA02fG	GGGGACAAGTTTGTACAAAAAAGCAGGCTCTAGAAAGCAAAGCAATGGCACC
	05wc1CsA02rG	GGGGACCACTTTGTACAAGAAAGCTGGGTCTAGATCCACCTCAGGTGAACC
TrLARb	05wc3EsA03fG	GGGGACAAGTTTGTACAAAAAAGCAGGCTCTAGAAAGCAATGGCACCAGCAGC
	05wc3EsA03rG	GGGGACCACTTTGTACAAGAAAGCTGGGTCTAGATCCACCTCAGGTGAACC
TrLARc	07wc1VsF06fG	GGGGACAAGTTTGTACAAAAAAGCAGGCTCTAGATAAAGCAATGGCACCAGC
	07wc1VsF06rG	GGGGACCACTTTGTACAAGAAAGCTGGGTCTAGATCCACCTCAGGTGAACC

The pPZP221:35S^2 binary vector was further modified to contain two expression cassettes within one T-DNA. The expression cassette from the binary vector pWM5 consisting of the ASSU promoter and the tob terminator was PCR-amplified with a proofreading DNA polymerase using oligonucleotide primers with the following sequences:

forward primer 5'-CCACCATGTTTGAAATTTATTATGTGTTTTTTTCCG-3';

reverse primer 5'-TAATCCCGGGTAAGGGCAGCCCATACAAATGAAGC-3'.

The PCR product was cut with BstXI and SmaI and cloned directionally into the equally cut pPZP221:35S^2 vector. Additionally, a GATEWAY® cloning cassette (Invitrogen) was introduced into the multicloning site in the 35S^2:rbcS expression cassette following the manufacturer's protocol. TrBANa was cloned into the ASSU:tob expression cassette, TrCHSa3 was amplified with the GATEWAY®-compatible primers (see Table 3) and cloned into the 35S^2:rbcS expression cassette. A transformation vector containing chimeric genes using full-length open reading frame cDNAs encoding white clover TrBANa protein in sense orientation under the control of the ASSU promoter and TrCHSc3 protein in sense orientation under the control of the CaMV 35S^2 promoter within the same T-DNA was generated (Figure 33).

EXAMPLE 5

Development of binary transformation vectors containing chimeric genes with a combination of 2 or more cDNA sequences from white clover TrCHSa3, TrCHSc, TrCHSf, TrCHSh, TrBANa, TrLARa, TrLARb and TrLARc

To alter the expression of the proteins involved in flavonoid biosynthesis, and more specifically condensed tannin biosynthesis to improve herbage quality and bloat-safety, a modular binary transformation vector system was used (Figure 34). The modular binary vector system enables simultaneous integration of up to seven transgenes the expression of which is controlled by individual promoter and terminator sequences into the plant genome (Goderis *et al.*, 2002).

The modular binary vector system consists of a pPZP200-derived vector (Hajdukiewicz *et al.*, 1994) backbone containing within the T-DNA a number of unique restriction sites recognised by homing endonucleases. The same

restriction sites are present in pUC18-based auxiliary vectors flanking standard multicloning sites. Expression cassettes comprising a selectable marker gene sequence or a cDNA sequence to be introduced into the plant under the control of regulatory sequences like promoter and terminator can be constructed in the auxiliary vectors and then transferred to the binary vector backbone utilising the homing endonuclease restriction sites. Up to seven expression cassettes can thus be integrated into a single binary transformation vector. The system is highly flexible and allows for any combination of cDNA sequence to be introduced into the plant with any regulatory sequence.

For example, a selectable marker cassette comprising the nos promoter and nos terminator regulatory sequences controlling the expression of the nptII gene was PCR-amplified using a proofreading DNA polymerase from the binary vector pKYLX71:35S[2] and directionally cloned into the AgeI and NotI sites of the auxiliary vector pAUX3166. Equally, other selectable marker cassettes can be introduced into any of the auxiliary vectors.

In another example, the expression cassette from the binary vector pWM5 consisting of the ASSU promoter and the tob terminator was PCR-amplified with a proofreading DNA polymerase and directionally cloned into the AgeI and NotI sites of the auxiliary vector pAUX3169. Equally, other expression cassettes can be introduced into any of the auxiliary vectors.

In yet another example, the expression cassette from the direct gene transfer vector pDH51 was cut using EcoRI and cloned directly into the EcoRI site of the auxiliary vector pAUX3132.

TABLE 4

List of primers used to PCR-amplify plant selectable marker cassettes and the regulatory elements used to control the expression of TrCHSa3, TrCHSc, TrCHSf, TrCHSh, TrBANa, TrLARa, TrLARb and TrLARc genes

expression cassette	primer	primer sequence (5'>3')
nos::nptII-nos	forward	ATAATAACCGGTTGATCATGAGCGGAGAATTAAGGG
	reverse	ATAATAGCGGCCGCTAGTAACATAGATGACACCGCG

expression cassette	primer	primer sequence (5'>3')
35S::aacC1-35S	forward	AATAGCGGCCGCGATTTAGTACTGGATTTTGG
	reverse	AATAACCGGTACCCACGAAGGAGCATCGTGG
$35S^2$::rbcS	forward	ATAATAACCGGTGCCCGGGGATCTCCTTTGCC
	reverse	ATAATAGCGGCCGCATGCATGTTGTCAATCAATTGG
assu::tob	forward	TAATACCGGTAAATTTATTATGRGTTTTTTTCCG
	reverse	TAATGCGGCCGCTAAGGGCAGCCCATACAAATGAAGC

The expression cassettes were further modified by introducing a GATEWAY® cloning cassette (Invitrogen) into the multicloning site of the respective pAUX vector following the manufacturer's protocol. In a recombination reaction, the cDNAs encoding the open reading frame sequences were transferred from the entry vector obtained as described in Example 4 to the GATEWAY®-enabled pAUX vector. Any combination of the regulatory elements with cDNA sequences of TrCHSa3, TrCHSc, TrCHSf, TrCHSh, TrBANa, TrLARa, TrLARb and TrLARc can be obtained. One typical example is given in Figure 35 with expression cassettes for the nptII plant selectable marker, TrBANa, TrLARa and TrCHSa3.

Complete expression cassettes comprising any combination of regulatory elements and cDNA sequences to be introduced into the plant were then cut from the auxiliary vectors using the respective homing endonuclease and cloned into the respective restriction site on the binary vector backbone. After verification of the construct by nucleotide sequencing, the binary transformation vector comprising a number of expression cassettes was used to generate transgenic white clover plants.

EXAMPLE 6

Production by Agrobacterium-mediated transformation and analysis of transgenic white clover plants carrying chimeric white clover TrCHSa3, TrCHSc, TrCHSf, TrCHSh, TrBANa, TrLARa, TrLARb and TrLARc genes involved in flavonoid biosynthesis

A set of binary transformation vectors carrying chimeric white clover genes involved in flavonoid biosynthesis, and more specifically condensed tannin biosynthesis to improve herbage quality and bloat-safety, were produced as detailed in Examples 4 and 5.

Agrobacterium-mediated gene transfer experiments were performed using these transformation vectors.

The production of transgenic white clover plants carrying the white clover TrCHSa3, TrCHSc, TrCHSf, TrCHSh, TrBANa, TrLARa, TrLARb and TrLARc cDNAs, either singly or in combination, is described here in detail.

Preparation of *Agrobacterium*

Agrobacterium tumefaciens strain AGL-1 transformed with one of the binary vector constructs detailed in Example 6 were streaked on LB medium containing 50 µg/ml rifampicin and 50 µg/ml kanamycin and grown at 27 °C for 48 hours. A single colony was used to inoculate 5 ml of LB medium containing 50 µg/ml rifampicin and 50 µg/ml kanamycin and grown over night at 27 °C and 250 rpm on an orbital shaker. The overnight culture was used as an inoculum for 500 ml of LB medium containing 50 µg/ml kanamycin only. Incubation was over night at 27 °C and 250 rpm on an orbital shaker in a 2 l Erlenmeyer flask.

Preparation of white clover seeds

1 spoon of seeds (ca. 500) was placed into a 280 µm mesh size sieve and washed for 5 min under running tap water, taking care not to wash seeds out of sieve. In a laminar flow hood, seeds were transferred with the spoon into an autoclaved 100 ml plastic culture vessel. A magnetic stirrer (wiped with 70% EtOH) and ca. 30 ml 70% EtOH were added, and the seeds were stirred for 5 min. The EtOH was discarded and replaced by 50 ml 1.5% sodium hypochlorite. The seeds were stirred for an additional 45 - 60 min on a magnetic stirrer. The sodium hypochlorite was then discarded and the seeds rinsed 3 to 4 times with autoclaved

ddH_2O. Finally 30 ml of ddH_2O were added, and seeds incubated over night at 10 - 15°C in an incubator.

Agrobacterium-mediated transformation of white clover

The seed coat and endosperm layer of the white clover seeds prepared as above were removed with a pair of 18 G or 21 G needles. The cotyledons were cut from the hypocotyl leaving a ca. 1.5 mm piece of the cotyledon stalk. The cotyledons were transferred to a petridish containing ddH_2O. After finishing the isolation of clover cotyledons, ddH_2O in the petridish was replaced with *Agrobacterium* suspension (diluted to an OD_{600} = 0.2 - 0.4). The petridish was sealed with its lid and incubated for 40 min at room temperature.

After the incubation period, each cotyledon was transferred to paper towel using the small dissection needle, dried and plated onto regeneration medium RM73. The plates were incubated at 25°C with a 16h light/8h dark photoperiod. On day 4, the explants were transferred to fresh regeneration medium. Cotyledons transformed with *Agrobacterium* were transferred to RM73 containing cefotaxime (antibacterial agent) and gentamycin. The dishes were sealed with Parafilm and incubated at 25°C under a 16/8 h photoperiod. Explants were subcultured every three weeks for a total of nine weeks onto fresh RM 73 containing cefotaxime and gentamycin. Shoots with a green base were then transferred to root-inducing medium RIM. Roots developed after 1 – 3 weeks, and plantlets were transferred to soil when the roots were well established.

This process is shown in detail in Figure 36.

Preparation of genomic DNA for real-time PCR and analysis for the presence of transgenes

3 – 4 leaves of white clover plants regenerated on selective medium were harvested and freeze-dried. The tissue was homogenised on a Retsch MM300 mixer mill, then centrifuged for 10 min at 1700xg to collect cell debris. Genomic DNA was isolated from the supernatant using Wizard Magnetic 96 DNA Plant System kits (Promega) on a Biomek FX (Beckman Coulter). 5 µl of the sample (50 µl) were then analysed on an agarose gel to check the yield and the quality of the genomic DNA.

Genomic DNA was analysed for the presence of the transgene by real-time PCR using SYBR Green chemistry. PCR primer pairs (Table 4) were designed using MacVector (Accelrys) or PrimerExpress (ABI). The forward primer was located within the 35S[2] promoter region and the reverse primer within the transgene to amplify products of approximately 150 - 250 bp as recommended. The positioning of the forward primer within the 35S[2] promoter region guaranteed that endogenous genes in white clover were not detected.

TABLE 5

List of primers used for Real-time PCR analysis of white clover plants transformed with chimeric white clover genes involved in condensed tannin biosynthesis

construct	primer 1 (forward), 5'->3'	primer 2 (reverse), 5'->3'
pPZP221TrCHSa3	CATTTCATTTGGAGAGGACACGC	AACACGGTTTGGTGGATTTGC
pPZP221TrCHSc	TTGGAGAGGACACGCTGAAATC	ACAAGTTGGTGAGGGAATGCC
pPZP221TrCHSf	CATTTCATTTGGAGAGGACACGC	TCGTTGCCTTTCCCTGAGTAGG
pPZP221TrCHSh	TCATTTGGAGAGGACACGCTG	CGGTCACCATTTTTTTGTTGGAGG
pPZP221TrBANa	TTGGAGAGGACACGCTGAAATC	CAACAAAACCAGTGCCACC
pPZP221TrLARa	ATGACGCACAATCCCACTATCC	AGCCTTAGAAGAGAGAAGAGGTCC
pPZP221TrLARb	ATGACGCACAATCCCACTATCC	AGCCTTAGAAGAGAGAAGAGGTCC
pPZP221TrLARc	ATGACGCACAATCCCACTATCC	AGCCTTAGAAGAGAGAAGAGGTCC

5 µl of each genomic DNA sample was run in a 50 µl PCR reaction including SYBR Green on an ABI 7700 (Applied Biosystems) together with samples containing DNA isolated from wild type white clover plants (negative control), samples containing buffer instead of DNA (buffer control) and samples containing the plasmid used for transformation (positive plasmid control). Cycling conditions used were 2 min. at 50 °C, 10 min. at 95 °C and then 40 cycles of 15 sec. at 95 °C, 1 min. at 60 °C.

Preparation of genomic DNA and analysis of DNA for presence and copy number of transgene by Southern hybridisation blotting

Genomic DNA for Southern hybridisation blotting was obtained from leaf material of white clover plants following the CTAB method. Southern hybridisation blotting experiments were performed following standard protocols as described in Sambrook *et al.* (1989). In brief, genomic DNA samples were digested with appropriate restriction enzymes and the resulting fragments separated on an agarose gel. After transfer to a membrane, a cDNA fragment representing a transgene or selectable marker gene was used to probe the size-fractionated DNA fragments. Hybridisation was performed with either radioactively labelled probes or using the non-radioactive DIG labelling and hybridisation protocol (Boehringer) following the manufacturer's instructions.

Plants were obtained after transformation with all chimeric constructs and selection on medium containing gentamycin. Details of plant analysis are given in Table 5 and Figures 37, 38 and 39.

TABLE 5

Transformation of white clover with binary transformation vectors comprising cDNAs of white clover genes involved in condensed tannin biosyntheses, selection and molecular analysis of regenerated plants.

construct	cotyledons transformed	selection into RIM	soil	QPCR-positive	Southern	copy number range
pPZP221-35S2::TrCHSa3	2358	135	32	23	n/d	
pPZP221-35S2::TrCHSc	3460	89	41	27	n/d	
pPZP221-35S2::TrCHSf	3931	113	44	27	n/d	
pPZP221-35S2::TrCHSh	3743	79	37	30	n/d	
pPZP221-35S2::TrBANa	2315	144	50	38	7	1 to 4
pPZP221-35S2::TrLARa	2487	88	45	38	n/d	
pPZP221-35S2::TrLARb	3591	133	47	47	5	1 to 3
pPZP221-35S2::TrLARc	2835	96	32	29	n/d	

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Finally, it is to be understood that various alterations, modifications and/or additions may be made without departing from the spirit of the present invention as outlined herein.

CLAIMS

1. A substantially purified or isolated nucleic acid or nucleic acid fragment encoding a condensed tannin biosynthetic enzyme selected from the group consisting of chalcone synthase (CHS), CHS-like, dihydroflavonol 4-reductase (BAN), BAN-like, leucoanthocyanidine reductase (LAR) and LAR-like, or a functionally active fragment or variant of such a polypeptide, from a clover (*Trifolium*), medic (*Medicago*), ryegrass (*Lolium*) or fescue (*Festuca*) species.

2. A nucleic acid or nucleic acid fragment according to Claim 1, wherein said nucleic acid or nucleic acid fragment is from white clover (*Trifolium repens*) or perennial ryegrass (*Lolium perenne*).

3. A nucleic acid or nucleic acid fragment according to Claim 1, encoding a CHS polypeptide or CHS-like polypeptide and including a nucleotide sequence selected from the group consisting of (a) sequences shown in Figures 2, 6, 10 and 14 hereto (Sequence ID Nos. 1, 3, 5 and 7, respectively); (b) complements of the sequences recited in (a); (c) sequences antisense to the sequences recited in (a) and (b); and (d) functionally active fragments and variants of the sequences recited in (a), (b) and (c); and (e) RNA sequences corresponding to the sequences recited in (a), (b), (c) and (d).

4. A nucleic acid or nucleic acid fragment according to Claim 1, encoding a BAN polypeptide or BAN-like polypeptide and including a nucleotide sequence selected from the group consisting of (a) sequence shown in Figure 18 hereto (Sequence ID No. 9); (b) complements of the sequence recited in (a); (c) sequences antisense to the sequences recited in (a) and (b); and (d) functionally active fragments and variants of the sequences recited in (a), (b) and (c); and (e) RNA sequences corresponding to the sequences recited in (a), (b), (c) and (d).

5. A nucleic acid or nucleic acid fragment according to Claim 1, encoding a LAR polypeptide or LAR-like polypeptide and including a nucleotide sequence selected from the group consisting of (a) sequences shown in Figures 22, 26 and 30 hereto (Sequence ID Nos. 11, 13 and 15, respectively); (b) complements of the sequences recited in (a); (c) sequences antisense to the sequences recited in (a) and (b); and (d) functionally active fragments and variants

of the sequences recited in (a), (b) and (c); and (e) RNA sequences corresponding to the sequences recited in (a), (b), (c) and (d).

6. A construct including one or more nucleic acid or nucleic acid fragments according to any one of claims 1 to 5.

7. A construct according to claim 6 including nucleic acids or nucleic acid fragments encoding both CHS or CHS-like and BAN or BAN-like polypeptides.

8. A construct according to claim 6 including nucleic acids or nucleic acid fragments encoding both CHS or CHS-like and LAR or LAR-like polypeptides.

9. A construct according to claim 6 including nucleic acids or nucleic acid fragments encoding both LAR or LAR-like and BAN or BAN-like polypeptides.

10. A construct according to claim 6 including nucleic acids or nucleic acid fragments encoding all three of CHS or CHS-like, LAR or LAR-like and BAN or BAN-like polypeptides.

11. A construct according to any one of claims 6 to 10 wherein the one or more nucleic acids or nucleic acid fragments are operably linked to one or more regulatory elements, such that the one or more nucleic acids or nucleic acid fragments are expressed.

12. A construct according to Claim 11, wherein the one or more regulatory elements include a promoter and a terminator, said promoter, nucleic acid or nucleic acid fragment and terminator being operatively linked.

13. A plant cell, plant, plant seed or other plant part, including a construct according to any one of claims 6 to 12.

14. A plant, plant seed or other plant part derived from a plant cell or plant according to Claim 13.

15. A method of modifying one or more of condensed tannin biosynthesis; protein binding, metal chelation; anti oxidation; UV-light absorption; pigment production; or plant defence to a biotic stress; in a plant, said method including introducing into said plant an effective amount of a nucleic acid or nucleic acid fragment according to any one or claims 1 to 5 or a construct according or any one of claims 6 to 12.

16. A method according to claim 15 wherein said method includes introducing into said plant effective amounts of nucleic acids or nucleic acid fragments encoding both CHS or CHS-like and BAN or BAN-like polypeptides.

17. A method according to claim 15 wherein said method includes introducing into said plant effective amounts of nucleic acids or nucleic acid fragments encoding both CHS or CHS-like and LAR or LAR-like polypeptides

18. A method according to claim 15 wherein said method includes introducing into said plant effective amounts of nucleic acids or nucleic acid fragments encoding both LAR or LAR-like and BAN or BAN-like polypeptides.

19. A method according to claim 15 wherein said method includes introducing into said plant effective amounts of nucleic acids or nucleic acid fragments encoding all three of CHS or CHS-like, BAN or BAN-like and LAR or LAR-like polypeptides.

20. A method according to any one of claims 15 to 19 wherein the method is modifying plant defence to biotic stress and the biotic stress is selected from the group consisting of viruses, micro-organisms, insects and fungal pathogens.

21. A method of modifying forage quality of a plant by disrupting protein foam and/or conferring protection from rumen pasture bloat, said method including introducing into said plant an effective amount of a nucleic acid fragment according to any one of claims 1 to 5 or a construct according to any one of claims 6 to 12.

22. A method according to claim 21 wherein said method includes introducing into said plant effective amounts of nucleic acids or nucleic acid fragments encoding both CHS or CHS-like and BAN or BAN-like polypeptides.

23. A method according to claim 21 wherein said method includes introducing into said plant effective amounts of nucleic acids or nucleic acid fragments encoding both CHS or CHS-like and LAR or LAR-like polypeptides

24. A method according to claim 21 wherein said method includes introducing into said plant effective amounts of nucleic acids or nucleic acid fragments encoding both LAR or LAR-like and BAN or BAN-like polypeptides.

25. A method according to claim 21 wherein said method includes introducing into said plant effective amounts of nucleic acids or nucleic acid fragments encoding all three of CHS or CHS-like, BAN or BAN-like and LAR or LAR-like polypeptides.

26. Use of a nucleic acid or nucleic acid fragment according to any one of claims 1 to 5, and/or nucleotide sequence information thereof, and/or single nucleotide polymorphisms thereof as a molecular genetic marker.

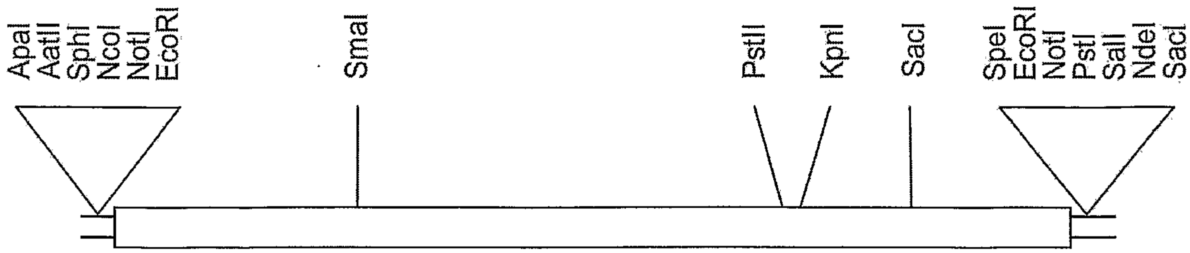
27. A substantially purified or isolated polypeptide from a clover (*Trifolium*), medic (*Medicago*), ryegrass (*Lolium*) or fescue (*Festuca*) species, selected from the group consisting of CHS and CHS-like, BAN and BAN-like and LAR and LAR-like; and functionally active fragments and variants thereof.

28. A polypeptide according to Claim 27, wherein said polypeptide is from white clover (*Trifolium repens*) or perennial ryegrass (*Lolium perenne*).

29. A polypeptide according to Claim 27, wherein said polypeptide is CHS or CHS-like and includes an amino acid sequence selected from the group consisting of sequences shown in Figures 3, 7, 11 and 15 hereto (Sequence ID Nos. 2, 4, 6 and 8, respectively); and functionally active fragments and variants thereof.

30. A polypeptide according to Claim 27, wherein said polypeptide is BAN or BAN-like and includes an amino acid sequence selected from the group consisting of sequence shown in Figure 19 hereto (Sequence ID No. 10); and functionally active fragments and variants thereof.

31. A polypeptide according to Claim 27, wherein said polypeptide is LAR or LAR-like and includes an amino acid sequence selected from the group consisting of sequences shown in Figures 23, 27 and 31 hereto (Sequence ID Nos. 12, 14 and 16, respectively); and functionally active fragments and variants thereof.



TrCHSa3

FIGURE 1

```
   1 GAATTCACTA GTGATTAAGC AGTGGTAACA ACGCAGAGTA CGCGGGGAAC
  51 AAAAACAACT ACGCATATTA TATATATATA TATATAGTCT ATAATTGAAA
 101 GAAACTGCTA AAGATATTAT TAAGATATGG TGAGTGTAGC TGAAATTCGC
 151 AAGGCTCAGA GGGCTGAAGG CCCTGCAACC ATTTTGGCCA TTGGCACTGC
 201 AAATCCACCA AACCGTGTTG AGCAGAGCAC ATATCCTGAT TTCTACTTCA
 251 AAATTACAAA CAGTGAGCAC AAGACTGAGC TCAAAGAGAA GTTCCAACGC
 301 ATGTGTGACA AATCCATGAT CAAGAGCAGA TACATGTATC TAACAGAAGA
 351 GATTTTGAAA GAAAATCCTA GTCTTTGTGA ATACATGGCA CCTTCATTGG
 401 ATGCTAGGCA AGACATGGTG GTGGTTGAGG TACCTAGACT TGGGAAGGAG
 451 GCTGCAGTCA AGGCCATTAA AGAATGGGGT CAACCAAAGT CAAAGATTAC
 501 TCACTTAATC TTTTGCACCA CAAGTGGTGT TGACATGCCT GGTGCTGATT
 551 ACCAACTCAC AAAACTCTTA GGTCTTCGCC CATATGTGAA AAGGTATATG
 601 ATGTACCAAC AAGGTTGTTT TGCAGGAGGC ACGGTGCTTC GTTTGGCAAA
 651 AGATTTGGCC GAGAACAACA AAGGTGCTCG TGTGCTAGTT GTTTGTTCTG
 701 AAGTCACCGC AGTCACATTT CGCGGCCCCA GTGATACTCA CTTGGACAGT
 751 CTTGTTGGAC AAGCATTGTT TGGAGATGGA GCCGCTGCAC TAATTGTTGG
 801 TTCTGATCCA GTGCCTGAAA TTGAGAAACC AATATTTGAG ATGGTTTGGA
 851 CTGCACAAAC AATTGCTCCA GACAGTGAAG GTGCCATTGA TGGTCATCTT
 901 CGTGAAGCTG GGCTAACATT TCATCTTCTT AAAGATGTTC CTGGGATTGT
 951 ATCAAAGAAC ATTAATAAAG CATTGGTTGA GGCTTTCCAA CCATTAGGAA
1001 TTTCTGACTA CAACTCAATC TTTTGGATTG CACACCCGGG TGGACCTGCA
1051 ATTCTTGATC AAGTAGAACA AAAGCTAGCC TTGAAGCCCG AAAAGATGAG
1101 GGCCACGAGG GAAGTTCTAA GTGAATATGG AAACATGTCA AGCGCATGTG
1151 TATTGTTCAT CTTAGATGAG ATGCGGAAGA AATCGGCTCA AAATGGACTT
1201 AAGACAACTG GAGAAGGACT TGATTGGGGT GTGTTGTTCG GCTTCGGACC
1251 AGGACTTACC ATTGAAACCG TTGTTCTTCG TAGCGTGGCT ATATAAGATG
1301 TGTGATTGTT TTTATTTTAA TGTATTACTT TTAATCTTGC TGCCTTGAAT
1351 TTCGATTTAA GAATAAATAA ATATATCTTT TGATAAAAAA AAAAAAAAAA
1401 AAAAAAAAAA AAGTACTCTG CGTTGTTACC ACTGCTTAAT CGAATTC
```

FIGURE 2

```
  1  MVSVAEIRKA QRAEGPATIL AIGTANPPNR VEQSTYPDFY FKITNSEHKT
 51  ELKEKFQRMC DKSMIKSRYM YLTEEILKEN PSLCEYMAPS LDARQDMVVV
101  EVPRLGKEAA VKAIKEWGQP KSKITHLIFC TTSGVDMPGA DYQLTKLLGL
151  RPYVKRYMMY QQGCFAGGTV LRLAKDLAEN NKGARVLVVC SEVTAVTFRG
201  PSDTHLDSLV GQALFGDGAA ALIVGSDPVP EIEKPIFEMV WTAQTIAPDS
251  EGAIDGHLRE AGLTFHLLKD VPGIVSKNIN KALVEAFQPL GISDYNSIFW
301  IAHPGGPAIL DQVEQKLALK PEKMRATREV LSEYGNMSSA CVLFILDEMR
351  KKSAQNGLKT TGEGLDWGVL FGFGPGLTIE TVVLRSVAI
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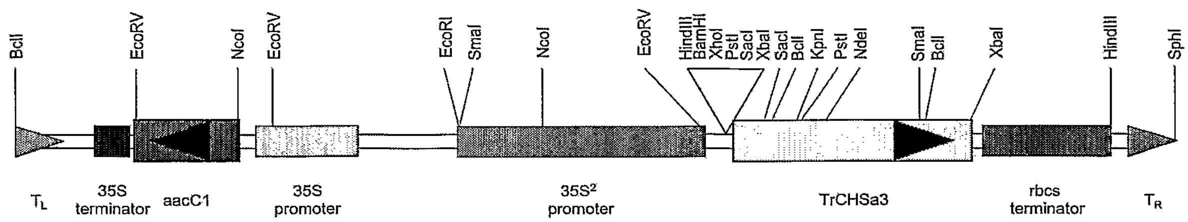
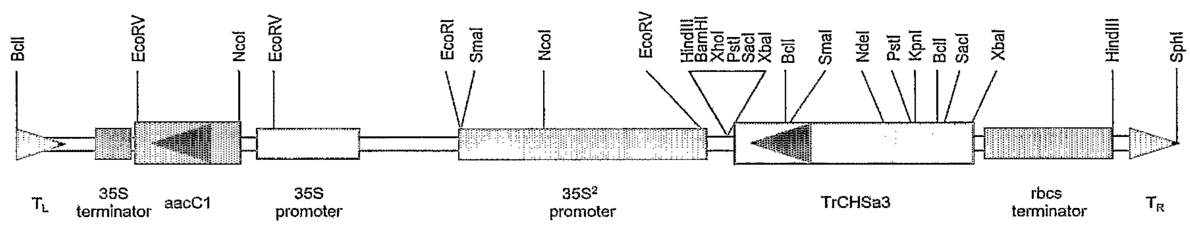
FIGURE 3

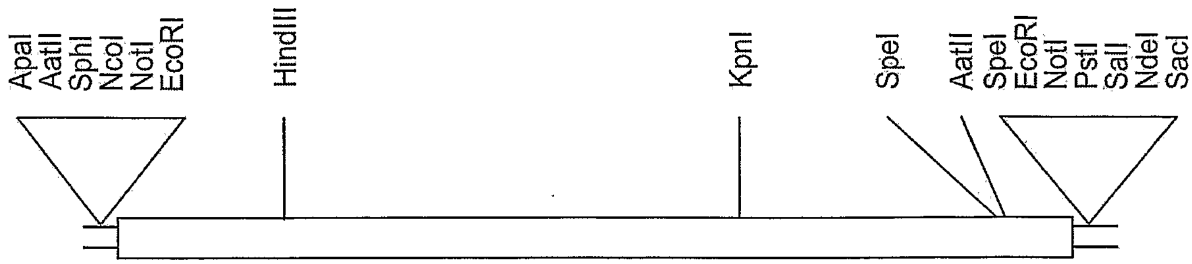


pPZP221:35S²TrCHSa3 sense



pPZP221:35S²TrCHSa3 anti

FIGURE 4



TrCHSc

FIGURE 5

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   1 GAATTCGATT AAGCAGTGGT AACAACGCAG AGTACGCGGG GATTCAATCT
  51 GTTGTGCATA AAATTCACTC ATTGCATAGA AAACCATACA CATTTGATCT
 101 TGCAAAGAAG AAATATGGGA GACGAAGGTA TAGTGAGAGG TGTCACAAAG
 151 CAGACAACCC CTGGGAAGGC TACTATATTG GCTCTTGGCA AGGCATTCCC
 201 TCACCAACTT GTGATGCAAG AGTGTTTAGT TGATGGTTAT TTTAGGGACA
 251 CTAATTGTGA CAATCCTGAA CTTAAGCAGA AACTTGCTAG ACTTTGTAAG
 301 ACAACCACGG TAAAAACAAG GTATGTTGTT ATGAATGAGG AGATACTAAA
 351 GAAATATCCA GAACTTGTTG TCGAAGGCGC CTCAACTGTA AAACAACGTT
 401 TAGAGATATG TAATGAGGCA GTAACACAAA TGGCAATTGA AGCTTCCCAA
 451 GTTTGCCTAA AGAATTGGGG TAGATCCTTA TCGGACATAA CTCATGTGGT
 501 TTATGTTTCA TCTAGTGAAG CTAGATTACC CGGTGGTGAC CTATACTTGT
 551 CAAAAGGACT AGGACTAAAC CCTAAAATTC AAAGAACCAT GCTCTATTTC
 601 TCTGGATGCT CGGGAGGCGT AGCCGGCCTT CGCGTTGCGA AAGACGTAGC
 651 TGAGAACAAC CCTGGAAGTA GAGTTTTGCT TGCTACTTCG GAAACTACAA
 701 TTATTGGATT CAAGCCACCA AGTGTTGATA GACCTTATGA TCTTGTTGGT
 751 GTGGCACTCT TTGGAGATGG TGCTGGTGCA ATGATAATTG GCTCAGACCC
 801 GGTATTTGAA ACTGAGACAC CATTGTTTGA GCTGCATACT TCAGCTCAGG
 851 AGTTTATACC AGACACCGAG AAGAAAATTG ATGGGCGGCT GACGGAGGAG
 901 GGCATAAGTT TCACACTAGC AAGGGAACTT CCGCAGATAA TCGAAGACAA
 951 TGTTGAGGGA TTCTGTAATA AACTAATTGA TGTTGTTGGG TTGGAGAATA
1001 AGGAGTACAA TAAGTTGTTT TGGGCTGTGC ATCCAGGTGG GCCTGCGATA
1051 TTGAATCGCG TGGAGAAGCG GCTTGAGTTG TCGCCGCAGA AGCTGAATGC
1101 TAGTAGAAAA GCTCTAATGG ATTATGGAAA TGCTAGCAGC AATACTATTG
1151 TTTATGTGCT GGAATATATG CTAGAAGAGG AAAAGAAGAT TAAAAAGGCG
1201 GGTGGAGGAG ATTCTGAATG GGGATTGATA CTTGCTTTTG GACCTGGAAT
1251 TACTTTTGAG GGGATTCTAG CAAGGAACTT GTGTGCATGA AGTCTTATAC
1301 AATTGTGATG CATGACTTAT ACTCTTATTT CTACTAATTA TTATATTAAG
1351 CAAATTCAGA ACTTTTAAGT AATGATTTAA TGAAGAATAC TTATAGTATA
1401 TTGACTTTAT TCACTTTCAA AGCAAGTTTA TGATCCTAAG ACATGGTAGA
1451 ACTTGAGCAT GTGGAATAGT TGTAACAAAA ACTCTAAGCA AATAGAGACT
1501 TTATGTAGTA TAAAGCATTT CCAGACATGA TAAATAATGG TACCTCAGAA
1551 CATAAAATAT ATTTAGCTAT CTTTCATCCC CAACTTTACA CATCCACCAA
1601 GGTACAGAAT AAGCATATGT CAACACAAAA TGTACTCTAA GTCTAACATG
1651 AGTAACCAAA CATGATGCCT GATTAAGTTA AAAGAAAAGA AAATCTGAGG
1701 GCATAGATCT TCAATCACAC CACTCCAGAG GGAAGGCGTA GAACAAGCTG
```

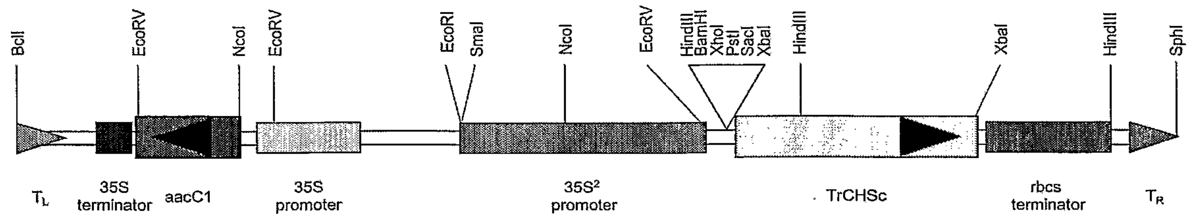
FIGURE 6

1751 TCCGCCGAAA ACACTGCAAT TCAATAAATA TCATTAGGAC AACAGTGCAG
1801 AGTCATGCGG GAAATGTCTT AAGTCACTGT ACTAAAAATA TAGGATTATA
1851 TTATGAACTA TACTAACCTT TTCACATAAT AGTAACAGAA ATCAGCTAAG
1901 ATGAATGTCT GGACAATTTC TGAGATAAGA ACCATGACGG CCATAAGCCA
1951 TACCCCAAGG CAACCAATAA ATGTCCACGG GTATCTAACA CCTGTTGCAA
2001 GAAATAGTAA GTTATTAGGA GATGTGCGGT TACGAAATTC AAGCTACACA
2051 ACAAAAGGAG GCCAGAACAA CAGCAATCTT GTAACCAGAT GACAACAATA
2101 AAATGTAAAC TTAAAGAGAC CGAACACACA AACATTGCAA CTCAGATGGA
2151 ATTGCTGCCA TGTAACTAGT AGGAGATTTG GGACGTCAAA TCAGTATATT
2201 ATGCAAATAC AAGGTATGAC CGCCTTGTCT ATTGTAGCAT ACAACAAACG
2251 TACAGTGGGT TTGTCCCTCT CAAAATGGCA GGATCTTTAC AGCACAATAT
2301 TTGGTTTTGT CATACTTATA CCATAAAAAA AAAAAAAAAA AAAAAAAAAA
2351 AAAGTACTCT GCGTTGTTAC CACTGCTTAA TCACTAGT**GA** **ATTC**

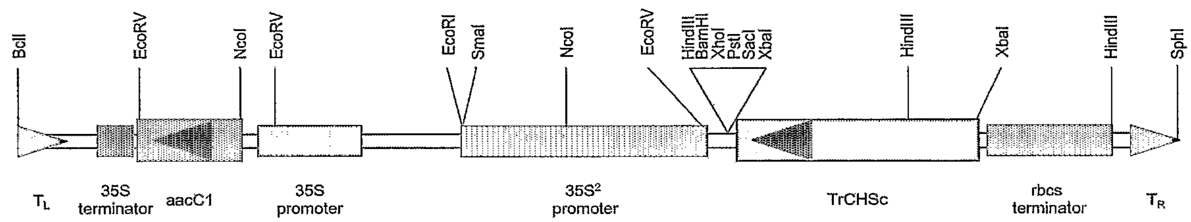
FIGURE 6 (cont.)

1 MGDEGIVRGV TKQTTPGKAT ILALGKAFPH QLVMQECLVD GYFRDTNCDN
51 PELKQKLARL CKTTTVKTRY VVMNEEILKK YPELVVEGAS TVKQRLEICN
101 EAVTQMAIEA SQVCLKNWGR SLSDITHVVY VSSSEARLPG GDLYLSKGLG
151 LNPKIQRTML YFSGCSGGVA GLRVAKDVAE NNPGSRVLLA TSETTIIGFK
201 PPSVDRPYDL VGVALFGDGA GAMIIGSDPV FETETPLFEL HTSAQEFIPD
251 TEKKIDGRLT EEGISFTLAR ELPQIIEDNV EGFCNKLIDV VGLENKEYNK
301 LFWAVHPGGP AILNRVEKRL ELSPQKLNAS RKALMDYGNA SSNTIVYVLE
351 YMLEEEKKIK KAGGGDSEWG LILAFGPGIT FEGILARNLC A

FIGURE 7

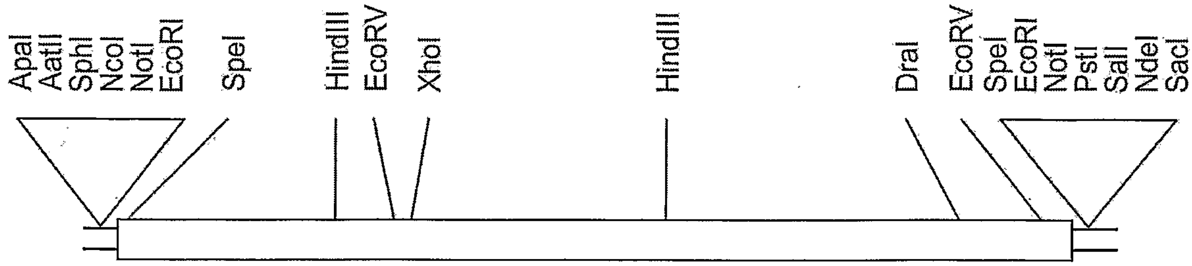


pPZP221:35S²TrCHSc sense



pPZP221:35S²TrCHSc anti

FIGURE 8



TrCHSf

FIGURE 9

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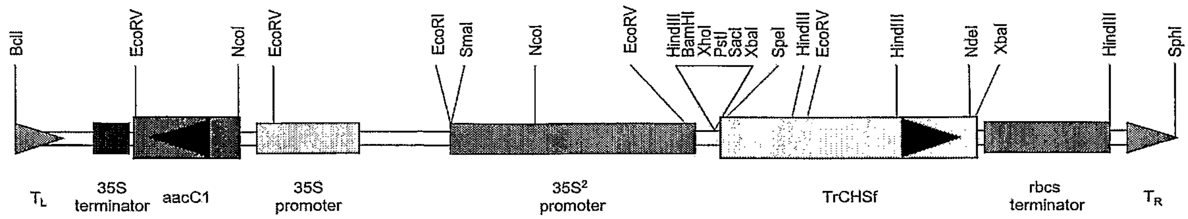
   1 GAATTCGATT AAGCAGTGGT AACAACGCAG AGTACGCGGG ACTAAGCCTT
  51 GATTCATTGT TTGTTTCCAT AACACAAGAA CTAGTGTTTG CTTGAATCTT
 101 AAGAAAAAAT GCCTCAAGGT GATTTGAATG GAAGTTCCTC GGTGAATGGA
 151 GCACGTGCTA GACGTGCTCC TACTCAGGGA AAGGCAACGA TACTTGCATT
 201 AGGAAAGGCT TTCCCCGCCC AGGTCCTCCC TCAAGAGTGC TTGGTGGAAG
 251 GATTCATTCG CGACACTAAG TGTGACGATA CTTATATTAA GGAGAAATTG
 301 GAGCGTCTTT GCAAAAACAC AACTGTGAAA ACAAGATACA CAGTAATGTC
 351 AAAGGAGATC TTAGACAACT ATCCAGAGCT AGCCATAGAT GGAACACCAA
 401 CAATAAGGCA AAAGCTTGAA ATAGCAAATC CAGCAGTAGT TGAAATGGCA
 451 ACAAGAGCAA GCAAAGATTG CATCAAAGAA TGGGGAAGGT CACCTCAAGA
 501 TATCACACAC ATAGTCTATG TTTCCTCGAG CGAAATTCGT CTACCCGGTG
 551 GTGACCTTTA TCTTGCAAAT GAACTCGGCT TAAACAGCGA TGTTAATCGC
 601 GTAATGCTCT ATTTCCTCGG TTGCTACGGC GGTGTCACTG GCTTACGTGT
 651 CGCCAAAGAC ATCGCCGAAA ATAACCCTGG TAGTAGGGTG TTACTCACAA
 701 CATCCGAGAC CACTATTCTC GGTTTTCGAC CACCGAGTAA AGCTAGACCT
 751 TATGACCTCG TTGGCGCTGC ACTTTTCGGT GATGGCGCCG CTGCTGCAAT
 801 AATTGGAACA GACCCTATAT TGAATCAAGA ATCACCTTTC ATGGAATTGA
 851 ACCATGCAGT CCAAAAATTC TTGCCTGATA CACAAAATGT GATTGATGGT
 901 AGAATCACTG AAGAGGGTAT TAATTTTAAG CTTGGAAGAG ACCTTCCTCA
 951 AAAAATTGAA GACAATATTG AAGAATTTTG CAAGAAAATT ATGGCTAAAA
1001 GTGATGTTAA GGAATTTAAT GACTTATTTT GGGCTGTTCA TCCTGGTGGG
1051 CCAGCTATAC TCAATAAGCT AGAAAATATA CTCAAATTGA AAAGTGATAA
1101 ATTGGATTGT AGTAGGAAGG CATTAATGGA TTATGGAAAT GTTAGTAGCA
1151 ATACTATATT CTATGTGATG GAGTATATGA GAGATTATTT GAAGGAAGAT
1201 GGAAGTGAAG AATGGGGATT AGGATTGGCT TTTGGACCAG GGATTACTTT
1251 TGAAGGGGTT CTCCTCCGTA GCCTTTAATC TTGAAATAAT AATTCATATG
1301 AAATTACTTG TCTTAAGATT GTGATAGGAA GATGAATATG TATTGGATTA
1351 ATATTGATAT GGTGTTATTT TAAGTTGATT TTAAAAAAAG TTTATTAATA
1401 AAGTATGATG TAACAATTGT TGTTTGAATG TTAAAAGGGA AGTATACTAT
1451 TTTAAGTTCT TGACCATACT GATTTTTTCT TTACACATTT TCATATCTAA
1501 AATTGTTCTA TGATATCTTC ATTGTTGATA CTGTAATAAT ATAATATCTA
1551 ATTTGGCTGG CAAAATGAAA GATTTTTCAC CGAAAAAAAA AAAAAAAAAA
1601 AAAAAAAAAA AAGTACTCTG CGTTGTTACC ACTGCTTAAT CACTAGTGAA
1651 TTC
```

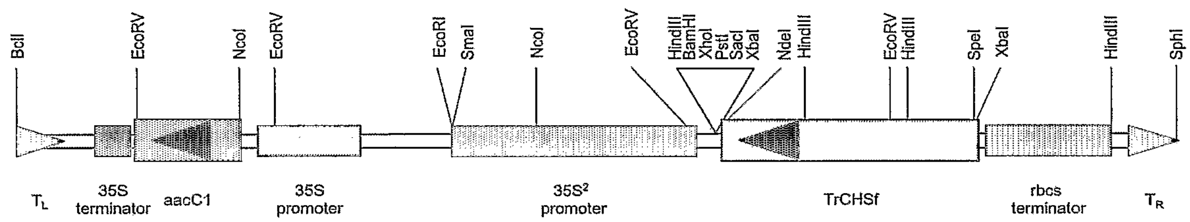
FIGURE 10

```
  1 MPQGDLNGSS SVNGARARRA PTQGKATILA LGKAFPAQVL PQECLVEGFI
 51 RDTKCDDTYI KEKLERLCKN TTVKTRYTVM SKEILDNYPE LAIDGTPTIR
101 QKLEIANPAV VEMATRASKD CIKEWGRSPQ DITHIVYVSS SEIRLPGGDL
151 YLANELGLNS DVNRVMLYFL GCYGGVTGLR VAKDIAENNP GSRVLLTTSE
201 TTILGFRPPS KARPYDLVGA ALFGDGAAAA IIGTDPILNQ ESPFMELNHA
251 VQKFLPDTQN VIDGRITEEG INFKLGRDLP QKIEDNIEEF CKKIMAKSDV
301 KEFNDLFWAV HPGGPAILNK LENILKLKSD KLDCSRKALM DYGNVSSNTI
351 FYVMEYMRDY LKEDGSEEWG LGLAFGPGIT FEGVLLRSL
```

FIGURE 11

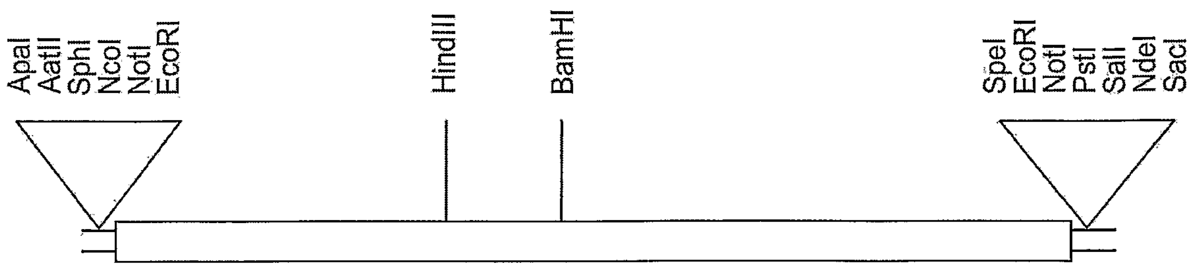


pPZP221:$35S^2$TrCHSf sense



pPZP221:$35S^2$TrCHSf anti

FIGURE 12



TrCHSh

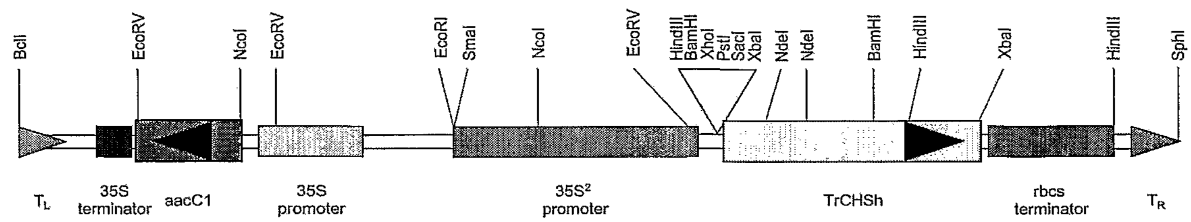
FIGURE 13

1 **GAATTC**ACTA GTGATTAAGC AGTGGTAACA ACGCAGAGTA CGCGGGGGAA
51 TCCACCAAAT CAACACCATT AATAACCTTC CAAATTCTCG TTACCTCACC
101 AAATCTCATT TTTCATTATA TATCTTGGGT ACATCTTTTG TTACCTCCAA
151 CAAAAAAATG GTGACCGTAG AAGAGATTCG TAACGCCCAA CGTTCAAATG
201 GCCCTGCCAC TATCTTAGCT TTTGGCACAG CCACTCCTTC TAACTGTGTC
251 ACTCAAGCTG ATTATCCTGA TTACTACTTT CGTATCACCA ACAGCGAACA
301 TATGACTGAT CTTAAGGAAA AATTCAAGCG GATGTGTGAT AGATCAATGA
351 TAAAGAAACG TTACATGCAC CTAACAGAAG ACTTTCTGAA GGAGAATCCA
401 AATATGTGTG AATACATGGC ACCATCACTA GATGTAAGAC GAGACATAGT
451 GGTTGTTGAA GTACCAAAGC TAGGTAAAGA AGCAGCAAAA AAAGCCATAT
501 GTGAATGGGG ACAACCAAAA TCCAAAATCA CACATCTTGT TTTCTGCACC
551 ACTTCCGGTG TTGACATGCC GGGAGCCGAT TACCAACTCA CCAAACTTTT
601 AGGCTTAAAA CCTTCTGTCA AGCGTCTCAT GATGTATCAA CAAGGTTGTT
651 TCGCTGGCGG CACAGTTCTC CGCTTAGCAA AAGACCTTGT TGAGAATAAC
701 AAAAATGCAA GAGTTCTTGT TGTTTGTTCT GAAATTACTG CGGTTACTTT
751 TCGTGGACCA TCGGATACTC ATCTTGATTC GCTCGTGGGA CAGGCGCTTT
801 TTGGTGATGG AGCCGCAGCA ATGATTATTG GTGCGGATCC TGATTTAACC
851 GTGGAGCGTC CGATTTTCGA GATTGTTTCG GCTGCTCAGA CTATTCTTCC
901 TGATTCTGAT GGCGCAATTG ATGGACATCT TCGTGAAGTG GGGCTCACTT
951 TTCATTTATT GAAAGATGTT CCGGGGATTA TTTCAAAGAA CATTGAAAAA
1001 AGTTTAGTTG AAGCTTTTGC GCCTATTGGG ATTAATGATT GGAACTCAAT
1051 ATTTTGGGTT GCACATCCAG GTGGACCGGC TATTTTAGAC CAGGTTGAAG
1101 AGAAACTCCA TCTTAAAGAG GAGAAACTCC GGTCCACCCG GCATGTGCTT
1151 AGTGAATATG GAAATATGTC AAGTGCATGT GTTTTATTTA TTTTGGATGA
1201 AATGAGAAAG AGGTCTAAAG AGGAAGGGAT GATTACAACT GGTGAAGGGT
1251 TGGAATGGGG TGTGTTGTTT GGGTTTGGAC CGGGTTTAAC TGTTGAAACC
1301 GTTGTGCTTC ATAGTGTTCC GGTTCAGGGT TGAATTTATT ATACATAGAT
1351 TGGAAAATAA AATTTGCCTG CCGAGAGATG TGAACTAACT TTGTAGGCAA
1401 GCTCAAATTA AAGTTTGAGA TAATATTGTG CTTTAGTTAT TATGGTATGT
1451 AATGTAATGT TTTTACTTTT TTCGAAATTC ATGTAATTTG ATATGTAAAG
1501 TAATATGTTT GGGTTGGAAT ATAATTATTT GTTAACTAAA AAAAAAAAAA
1551 AAAAAAAAAA AAAAAGTACT CTGCGTTGTT ACCACTGCTT AATC**GAATTC**

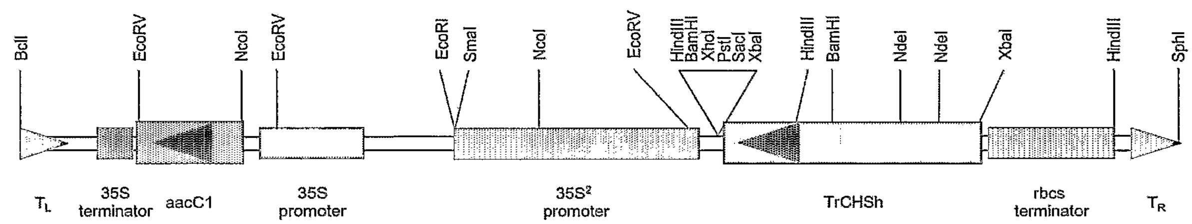
FIGURE 14

1	MVTVEEIRNA	QRSNGPATIL	AFGTATPSNC	VTQADYPDYY	FRITNSEHMT
51	DLKEKFKRMC	DRSMIKKRYM	HLTEDFLKEN	PNMCEYMAPS	LDVRRDIVVV
101	EVPKLGKEAA	KKAICEWGQP	KSKITHLVFC	TTSGVDMPGA	DYQLTKLLGL
151	KPSVKRLMMY	QQGCFAGGTV	LRLAKDLVEN	NKNARVLVVC	SEITAVTFRG
201	PSDTHLDSLV	GQALFGDGAA	AMIIGADPDL	TVERPIFEIV	SAAQTILPDS
251	DGAIDGHLRE	VGLTFHLLKD	VPGIISKNIE	KSLVEAFAPI	GINDWNSIFW
301	VAHPGGPAIL	DQVEEKLHLK	EEKLRSTRHV	LSEYGNMSSA	CVLFILDEMR
351	KRSKEEGMIT	TGEGLEWGVL	FGFGPGLTVE	TVVLHSVPVQ	G

FIGURE 15

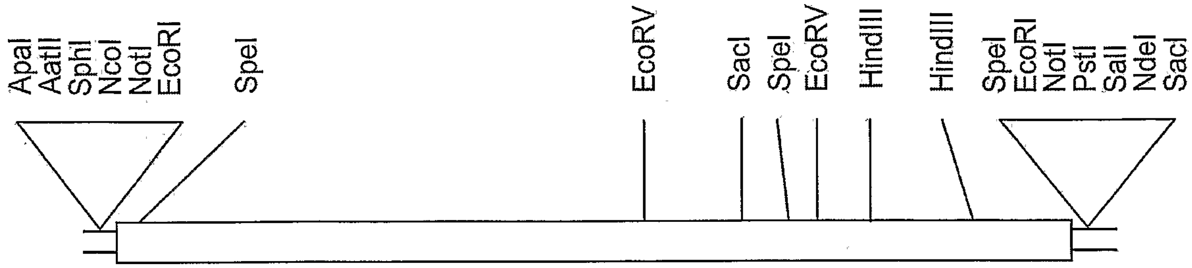


pPZP221:35S^{2}TrCHSh sense



pPZP221:35S^{2}TrCHSh anti

FIGURE 16



TrBANa

FIGURE 17

```
   1 GAATTCGATT AAGCAGTGGT AACAACGCAG AGTACGCGGG ATAAAAACTG
  51 CACTAGTGTG TATAAGTTTC TTGGTGAAAA AAGAGTTTGT AAATTAACAT
 101 CATGGCTAGT ATCAAACAAA TTGGAAACAA GAAAGCATGT GTGATTGGTG
 151 GCACTGGTTT TGTTGCATCT ATGTTGATCA AGCAGTTACT TGAAAAGGGT
 201 TATGCTGTTA ATACTACCGT TAGAGACCCA GATAGCCCTA AGAAAATATC
 251 TCACCTAGTG GCACTGCAAA GTTTGGGGGA ACTGAATCTA TTTAGAGCAG
 301 ACTTAACAGT TGAAGAAGAT TTTGATGCTC CTATAGCAGG ATGTGAACTT
 351 GTTTTTCAAC TTGCTACACC TGTGAACTTT GCTTCTCAAG ATCCTGAGAA
 401 TGACATGATA AAGCCAGCAA TCAAAGGTGT GTTGAATGTG TTGAAAGCAA
 451 TTGCAAGAGC AAAAGAAGTT AAAAGAGTTA TCTTAACATC TTCGGCAGCC
 501 GCGGTGACTA TAAATGAACT CAAAGGGACA GGTCATGTTA TGGATGAAAC
 551 CAACTGGTCT GATGTTGAAT TTCTCAACAC TGCAAAACCA CCCACTTGGG
 601 GTTATCCTGC CTCAAAAATG CTAGCTGAAA AGGCTGCATG GAAATTTGCT
 651 GAAGAAAATG ACATTGATCT AATCACTGTG ATACCTAGTT TAACAACTGG
 701 TCCTTCTCTC ACACCAGATA TCCCATCTAG TGTTGGCTTG GCAATGTCTC
 751 TAATAACAGG CAATGATTTT CTCATAAATG CTTTGAAAGG AATGCAGTTT
 801 CTGTCGGGTT CGTTATCCAT CACTCATGTT GAGGATATTT GCCGAGCTCA
 851 TATATTTCTT GCAGAGAAAG AATCAGCTTC TGGTAGATAC ATTTGCTGTG
 901 CTCACAATAC TAGTGTTCCC GAGCTTGCAA AGTTTCTCAA CAAACGATAT
 951 CCTCAGTATA AAGTTCCAAC TGAATTTGAT GATTGCCCCA GCAAGGCAAA
1001 GTTGATAATC TCTTCTGAAA AGCTTATCAA AGAAGGGTTC AGTTTCAAGC
1051 ATGGTATTGC CGAAACTTTC GACCAGACTG TCGAGTATTT TAAGACTAAG
1101 GGGGCACTGA AGAATTAGAT TTTGATATTT CTAATTCAAT AGCAAACTCT
1151 AAGCTTGTTA TGTGTTTGTG AAGTTCAGAG TGAAATATCA AATGAATAAG
1201 TGGAGAGAGC ACAATAAGAG GAGAGCACAA TAATTTTGGA AAAAAAAAAA
1251 AAAAAAAAAA AAAAAAAAGT ACTCTGCGTT GTTACCACTG CTTAATCACT
1301 AGTGAATTC
```

FIGURE 18

1 MASIKQIGNK KACVIGGTGF VASMLIKQLL EKGYAVNTTV RDPDSPKKIS
51 HLVALQSLGE LNLFRADLTV EEDFDAPIAG CELVFQLATP VNFASQDPEN
101 DMIKPAIKGV LNVLKAIARA KEVKRVILTS SAAAVTINEL KGTGHVMDET
151 NWSDVEFLNT AKPPTWGYPA SKMLAEKAAW KFAEENDIDL ITVIPSLTTG
201 PSLTPDIPSS VGLAMSLITG NDFLINALKG MQFLSGSLSI THVEDICRAH
251 IFLAEKESAS GRYICCAHNT SVPELAKFLN KRYPQYKVPT EFDDCPSKAK
301 LIISSEKLIK EGFSFKHGIA ETFDQTVEYF KTKGALKN

FIGURE 19

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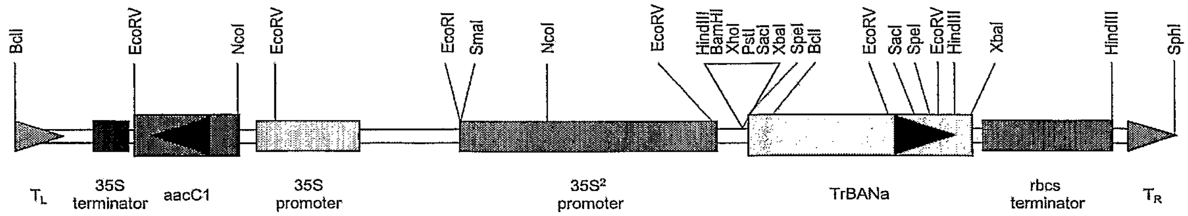
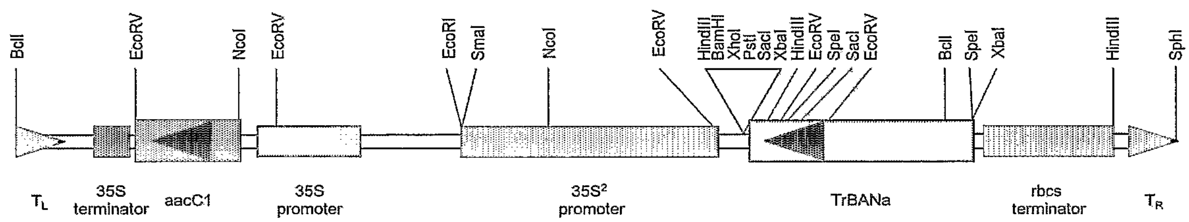


pPZP221:35S^{2}TrBANa sense



pPZP221:35S^{2}TrBANa anti

FIGURE 20

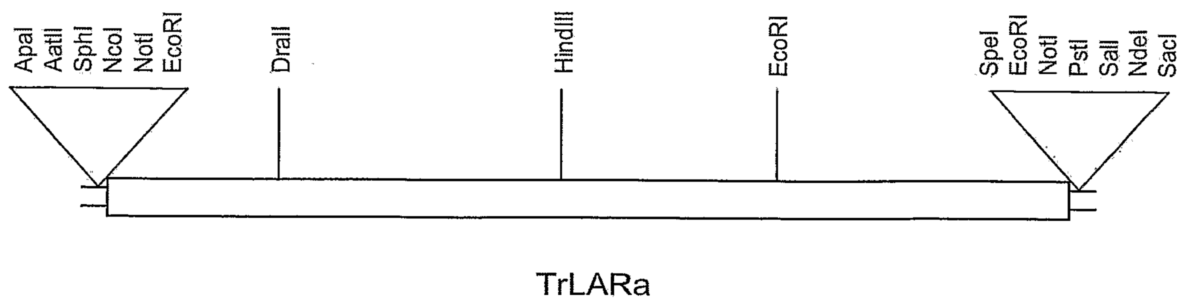

ApaI
AatII
SphI
NcoI
NotI
EcoRI
DraII
HindIII
EcoRI
SpeI
EcoRI
NotI
PstI
SalI
NdeI
SacI
TrLARa

FIGURE 21

```
   1 GAATTCGATT AAGCAGTGGT AACAACGCAG AGTACGCGGG GATACCAACA
  51 TTGTCACAAT TAACTCTAAA AGCAAAGCAA TGGCACCAGC AGCAACATCA
 101 TCACCAACCA CTCCTACTAC TACCAAGGGT CGTGTCCTAA TTGTTGGAGG
 151 AACAGGTTTC ATTGGAAAAT TTGTAACTGA GGCAAGTCTT TCCACAACAC
 201 ACCCAACCTA CTTGTTGGTT CGGCCAGGAC CTCTTCTCTC TTCTAAGGCT
 251 GCCACTATTA AGGCATTCCA AGAGAAAGGT GCCATTGTCA TTTATGGTCG
 301 GGTAAATAAT AAGGAGTTCA TGGAGATGAT TTTGAAAAAG TATGAGATAA
 351 ATGTAGTCAT TTCTGCAATA GGAGGCTCTG ATGGCTTGCT GGAACAGCTT
 401 ACTTTGGTGG AGGCCATGAA ATCTATTAAC ACCATTAAGA GGTTTTTGCC
 451 TTCGGAATTT GGTCACGATG TGGACAGAGC AAATCCTGTG GAACCTGGCC
 501 TAACAATGTA CAAACAGAAA CGTTTGGTTA GACGTGTGAT CGAAGAATCT
 551 GGTATACCAT ACACCTACAT CTGTTGCAAT TCGATCGCAT CTTGGCCGTA
 601 CTATGACAAT TGTCATCCAT CACAGCTTCC TCCACCGTTG GATCAATTAC
 651 ATATTTATGG TCATGGCGAT GTCAAAGCTT ACTTTGTTGA TGGCTATGAT
 701 ATTGGGAAAT TCACAATGAA GGTCATTGAT GATGAAAGAA CAATCAACAA
 751 AAATGTTCAT TTTCGACCTT CTAACAATTG TTATAGCATG AATGAGCTTG
 801 CTTCTTTGTG GGAAAACAAA ATTGCACGAA AAATTCCTAG AGTGATCGTC
 851 TCTGAAGACG ATCTTCTAGC AATAGCCGCA GAAAATTGCA TACCGGAAAG
 901 TGTCGTGGCA CCAATCACTC ATGATATATT CATCAATGGA TGTCAAGTTA
 951 ACTTCAAGAT AGATGGAATT CATGATGTTG AAATTGGCAC TCTATATCCT
1001 GGTGAATCGG TAAGAAGTTT GGAGGAATGC TATGAGAAAT TTGTTGTCAT
1051 GGCGGCTGAC AAGATTCATA AAGAAGAAAC TGGAGTTACC GCAGGTGGGG
1101 GCGGCACAAC GGCTATGGTA GAGCCGGTGC CAATCACAGC TTCCTGTTGA
1151 AAAGGTTCAC CTGAGGTGGA TATTCTTTTG AGTCATAAGA CATGTTGATT
1201 GTTGATGTTG TTTTCAAGAA TGTTTCATCA TTTCATGTGT TTTATTAATC
1251 CTAAGTACAA ATAATTGCTG TCTACGTACG TTCTTAGTTG CAAAAATTCT
1301 TGTTATTCTC TATTGAGGTA AAAGTCTTCA TGTTTACAAA AAAAAAAAAA
1351 AAAAAAAAAA AAAAAAAAGT ACTCTGCGTT GTTACCACTG CTTAATCACT
1401 AGTGAATTC
```

FIGURE 22

```
  1 MAPAATSSPT TPTTTKGRVL IVGGTGFIGK FVTEASLSTT HPTYLLVRPG
 51 PLLSSKAATI KAFQEKGAIV IYGRVNNKEF MEMILKKYEI NVVISAIGGS
101 DGLLEQLTLV EAMKSINTIK RFLPSEFGHD VDRANPVEPG LTMYKQKRLV
151 RRVIEESGIP YTYICCNSIA SWPYYDNCHP SQLPPPLDQL HIYGHGDVKA
201 YFVDGYDIGK FTMKVIDDER TINKNVHFRP SNNCYSMNEL ASLWENKIAR
251 KIPRVIVSED DLLAIAAENC IPESVVAPIT HDIFINGCQV NFKIDGIHDV
301 EIGTLYPGES VRSLEECYEK FVVMAADKIH KEETGVTAGG GGTTAMVEPV
351 PITASC
```

FIGURE 23

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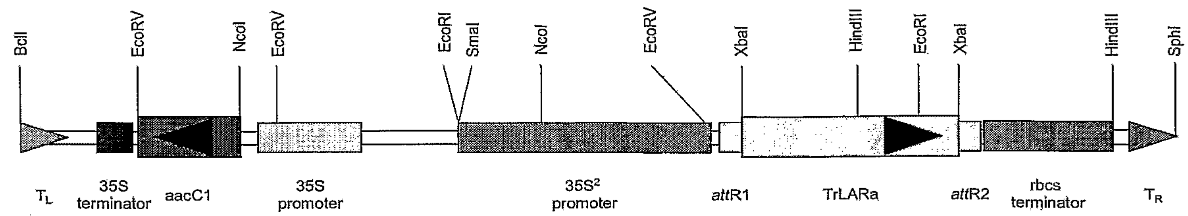

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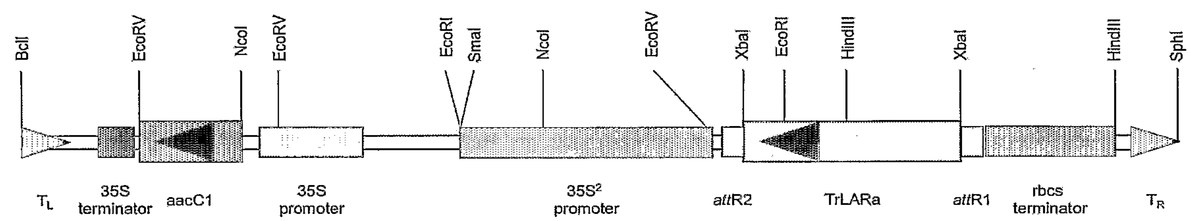


pPZP221:35S²TrLARa anti

FIGURE 24

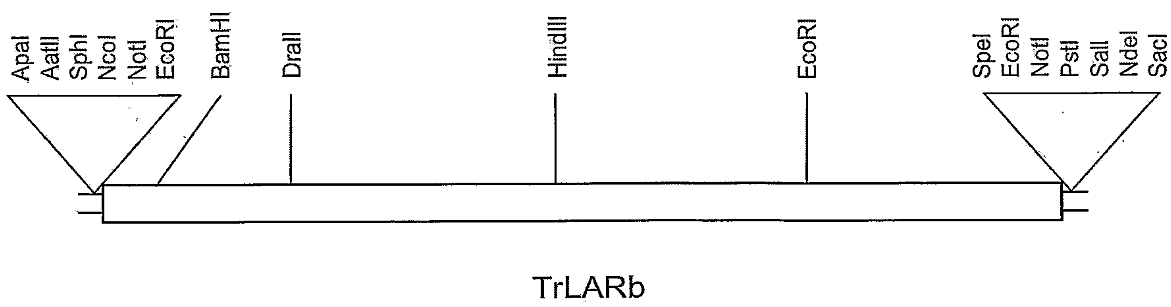

ApaI
AatII
SphI
NcoI
NotI
EcoRI
BamHI
DraII
HindIII
EcoRI
SpeI
EcoRI
NotI
PstI
SalI
NdeI
SacI
TrLARb

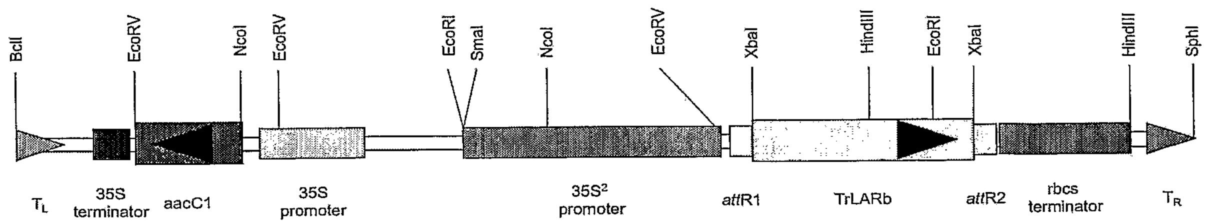
FIGURE 25

1 **GAATTC**GATT AAGCAGTGGT AACAACGCAG AGTACGCGGG AGGATCCTTC
51 CATTTTGCAT ACCAACATTG TCACAATTAA CTCTAAAAGC AAAGCAATGG
101 CACCAGCAGC AACATCATCA CCAACCACTC CTACTACTAC CAAGGGTCGT
151 GTCCTAATTG TTGGAGGAAC AGGTTTCATT GGAAAATTTG TAACTGAGGC
201 AAGTCTTTCC ACAACACACC CAACCTACTT GTTGGTTCGG CCAGGACCTC
251 TTCTCTCTTC TAAGGCTGCC ACTATTAAGG CATTCCAAGA GAAAGGTGCC
301 ATTGTCATTT ATGGTCGGGT AAATAATAAG GAGTTCATGG AGATGATTTT
351 GAAAAAGTAT GAGATAAATG TAGTCATTTC TGCAATAGGA GGCTCTGATG
401 GCTTGCTGGA ACAGCTTACT TTGGTGGAGG CCATGAAATC TATTAACACC
451 ATTAAGAGGT TTTTGCCTTC AGAATTTGGT CACGATGTGG ACAGAGCAAA
501 TCCTGTGGAA CCTGGCCTAA CAATGTACAA ACAGAAACGT TTGGTTAGAC
551 GTGTGATCGA AGAATCTGGT GTACCATACA CCTACATCTG TTGCAATTCG
601 ATCGCATCCT GGCCGTACTA TGACAATTGT CATCCATCAC AGCTTCCTCC
651 ACCGTTGGAT CAATTACATA TTTATGGTCA TGGCGATGTC AAAGCTTACT
701 TTGTTGATGG CTATGATATT GGGAAATTCA CAATGAAGGT CATTGATGAT
751 GAAAGAACAA TCAACAAAAA TGTTCATTTT CGACCTTCTA ACAATTGTTA
801 TAGCATGAAT GAGCTTGCTT CTTTGTGGGA AAACAAAATT GCACGAAAAA
851 TTCCTAGAGT GATCGTCTCT GAAGACGATC TTCTAGCAAT AGCCGCAGAA
901 AACTGCATAC CGGAAAGTGT TGTGGCATCA ATCACTCATG ATATATTCAT
951 CAATGGATGT CAAGTTAACT TCAAGGTAGA TGGAATTCAT GATGTTGAAA
1001 TTGGCACTCT ATATCCTGGT GAATCGGTAA GAAGTTTGGA GGAATGCTAT
1051 GAGAAATTTG TTGTCATGGC GGCTGACAAG ATTCATAAAG AAGAAACTGG
1101 AGTTACCGCA GGTGGGGGCG GCACAACGGC TATGGTAGAG CCGGTGCCAA
1151 TCACAGCTTC CTGTTGAAAA GGTTCACCTG AGGTGGATAT TCTTTTGAGT
1201 CATAAGACAT GTTGATTGTT GATGTTGTTT TCAAGAATGT TTCATCATTT
1251 CATGTGTTTT ATTAATCCTA AGTACAAATA ATTGCTGTCT ACGTACGTTC
1301 TTAGTTGCGA AAATTCTTGT TATTCTCTAT TGGGGTAAAA GTCTTCATGT
1351 TTATTGTAGT TGTGTTGGTT TTTCATATAT GCTATTTGCA ATAATGATTT
1401 TTGTGAAGCA CTTGTGGTGT ATTTACTTAC TACTGAAAAT AATGGTTACA
1451 CAAAATATAT AAAAAAATAA AAATAAGCAA AAAAAAAAAA AAAAAAAAAA
1501 AAAAAAAAAA GTACTCTGCG TTGTTACCAC TGCTTAATCA CTAGT**GAATT**
1551 **C**

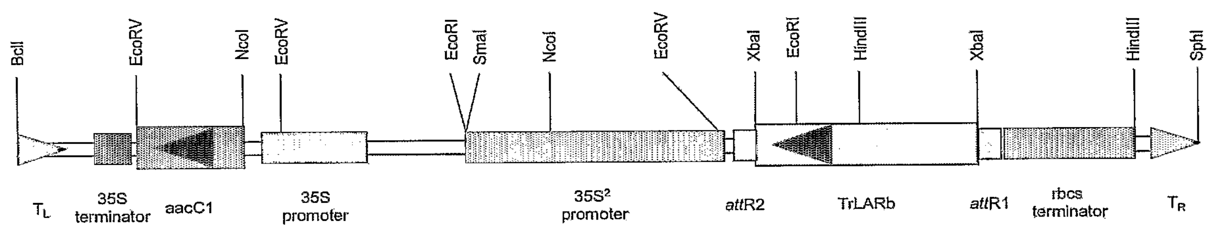
FIGURE 26

1	MAPAATSSPT	TPTTTKGRVL	IVGGTGFIGK	FVTEASLSTT	HPTYLLVRPG
51	PLLSSKAATI	KAFQEKGAIV	IYGRVNNKEF	MEMILKKYEI	NVVISAIGGS
101	DGLLEQLTLV	EAMKSINTIK	RFLPSEFGHD	VDRANPVEPG	LTMYKQKRLV
151	RRVIEESGVP	YTYICCNSIA	SWPYYDNCHP	SQLPPPLDQL	HIYGHGDVKA
201	YFVDGYDIGK	FTMKVIDDER	TINKNVHFRP	SNNCYSMNEL	ASLWENKIAR
251	KIPRVIVSED	DLLAIAAENC	IPESVVASIT	HDIFINGCQV	NFKVDGIHDV
301	EIGTLYPGES	VRSLEECYEK	FVVMAADKIH	KEETGVTAGG	GGTTAMVEPV
351	PITASC				

FIGURE 27

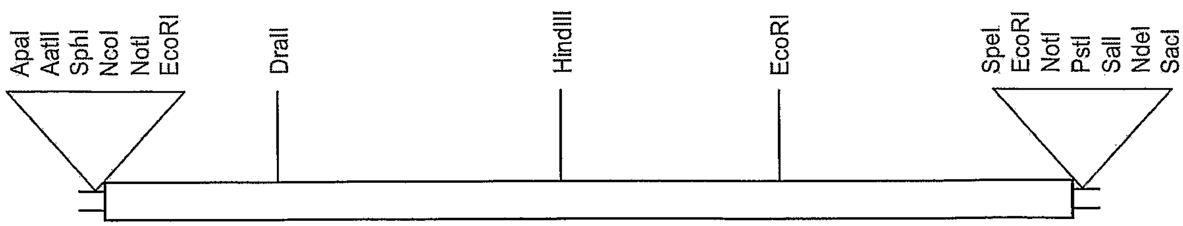


pPZP221:35S²TrLARb sense



pPZP221:35S²TrLARb anti

FIGURE 28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NotI
EcoRI
DraII
HindIII
EcoRI
SpeI
EcoRI
NotI
PstI
SalI
NdeI
SacI
TrLARc

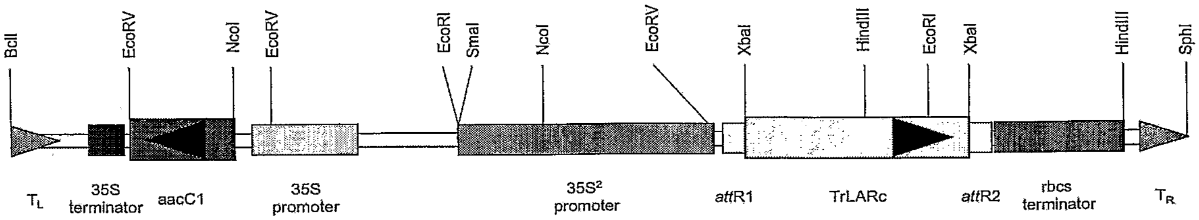
FIGURE 29

1 **GAATTC**GATT AAGCAGTGGT AACAACGCAG AGTACGCGGG GATACCAACA
51 TTGTCACAAT TAACTCTAAA AGTAAAGCAA TGGCACCAGC AGCAACATCA
101 TCACCAACCA CTCCCACTAC TACCAAGGGT CGTGTCCTAA TTGTTGGAGG
151 AACAGGTTTC ATTGGAAAAT TTGTAACTGA GGCAAGTCTT TCCACAACAC
201 ACCCAACCTA CTTGTTGGTT CGGCCAGGAC CTCTTCTCTC TTCTAAGGCT
251 GCCACTATTA AGGCATTCCA AGAGAAAGGT GCCATTGTCA TTTATGGTCG
301 GGTAAATAAT AAGGAGTTCA TGGAGATGAT TTTGAAAAAG TATGAGATAA
351 ATGTAGTCAT TTCTGCAATA GGAGGCTCTG ATGGCTTGCT GGAACAGCTT
401 ACTTTGGTGG AGGCCATGAA ATCTATTAAC ACCATTAAGA GGTTTTTGCC
451 TTCGGAATTT GGTCACGATG TGGACAGAGC AGATCCTGTG GAACCTGGCC
501 TAACAATGTA CAAACAGAAA CGTTTGGTTA GACGTGTGAT CGAAGAATCT
551 GGTATACCAT ACACCTACAT CTGTTGCAAT TCGATCGCAT CTTGGCCGTA
601 CTATGACAAT TGTCATCCAT CACAGCTTCC TCCACCGTTG GATCAATTAC
651 ATATTTATGG TCATGGCGAT GTCAAAGCTT ACTTTGTTGA TGGCTATGAT
701 ATTGGGAAAT TCACAATGAA GGTCATTGAT GATGAAAGAA CAATCAACAA
751 AAATGTTCAT TTTCGACCTT CTAACAATTG TTATAGCATG AATGAGCTTG
801 CTTCTTTGTG GGAAAACAAA ATTGCACGAA AAATTCCTAG AGTGATCGTC
851 TCTGAAGACG ATCTTCTAGC AATAGCCGCA GAAAATTGCA TACCGGAAAG
901 TGTCGTGGCA CCAATCACTC ATGATATATT CATCAATGGA TGTCAAGTTA
951 ACTTCAAGAT AGATGGAATT CATGATGTTG AAATTGGCAC TCTATATCCT
1001 GGTGAATCGG TAAGAAGTTT GGAGGAATGC TATGAGAAAT TTGTTGTCAT
1051 GGCGGCTGAC AAGATTCATA AAGAAGAAAC TGGAGTTACC GCAGGTGGGG
1101 GCGGCACAAC GGCTATGGTA GAGCCGGTGC CAATCACAGC TTCCTGTTGA
1151 AAAGGTTCAC CTGAGGTGGA TATTCTTTTG AGTCATAAGA CATGTTGATT
1201 GTTGATGTTG TTTTCAAGAA TGTTTCATCA TTTCATGTGT TTTATTAATC
1251 CTAAGTACAA ATAATTGCTG TCTACGTACG TTCTTAGTTG CAAAAATTCT
1301 TGTTATTCTC TATCAAAAAA AAAAAAAAAA AAAAAAAAAA AAAGTACTCT
1351 GCGTTGTTAC CACTGCTTAA TCACTAGT**GA ATTC**

FIGURE 30

1	MAPAATSSPT	TPTTTKGRVL	IVGGTGFIGK	FVTEASLSTT	HPTYLLVRPG
51	PLLSSKAATI	KAFQEKGAIV	IYGRVNNKEF	MEMILKKYEI	NVVISAIGGS
101	DGLLEQLTLV	EAMKSINTIK	RFLPSEFGHD	VDRADPVEPG	LTMYKQKRLV
151	RRVIEESGIP	YTYICCNSIA	SWPYYDNCHP	SQLPPPLDQL	HIYGHGDVKA
201	YFVDGYDIGK	FTMKVIDDER	TINKNVHFRP	SNNCYSMNEL	ASLWENKIAR
251	KIPRVIVSED	DLLAIAAENC	IPESVVAPIT	HDIFINGCQV	NFKIDGIHDV
301	EIGTLYPGES	VRSLEECYEK	FVVMAADKIH	KEETGVTAGG	GGTTAMVEPV
351	PITASC				

FIGURE 31



pPZP221:35S²TrLARc sense

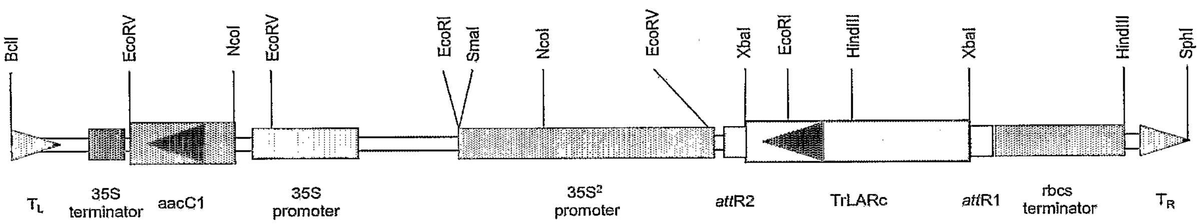


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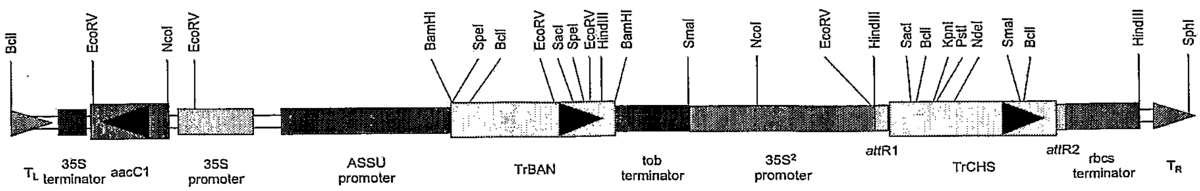
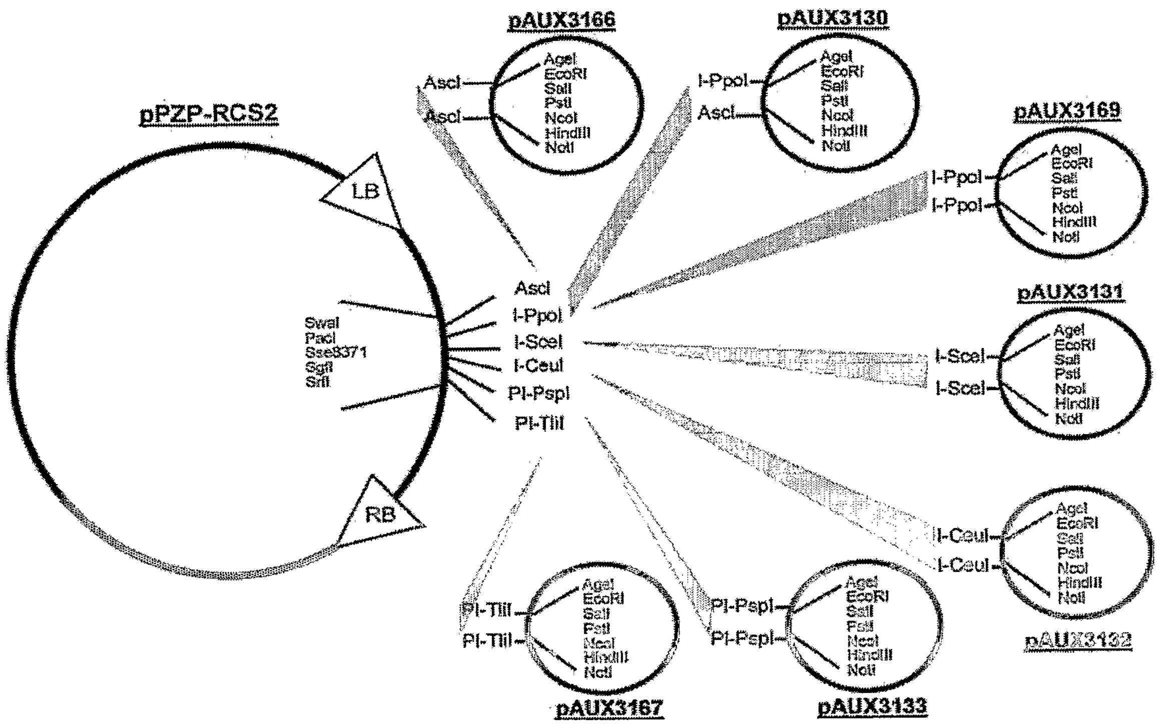
FIGURE 32



pPZP221:ASSU::BAN:35S^2::CHS

FIGURE 33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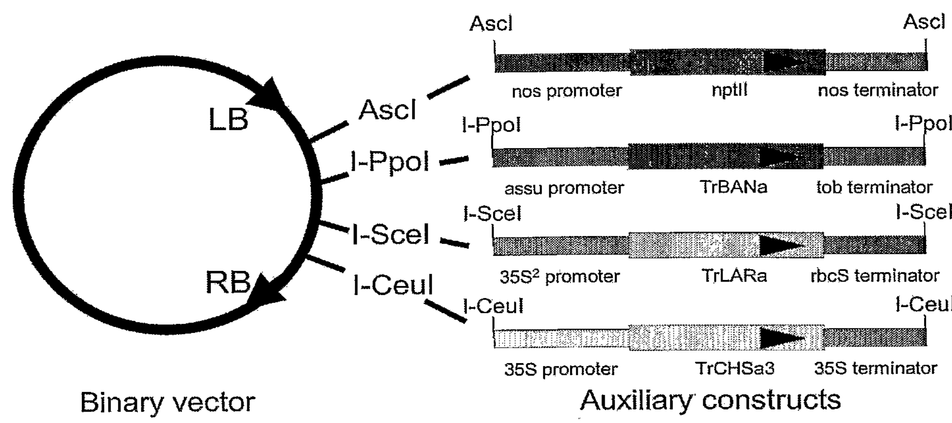
RB
SwaI
PacI
Sse8371
SgfI
SrfI
AscI
I-PpoI
I-SceI
I-CeuI
PI-PspI
PI-TliI
pAUX3166
pAUX3130
pAUX3169
pAUX3131
pAUX3132
pAUX3133
pAUX3167
AgeI
EcoRI
SalI
PstI
NcoI
HindIII
NotI

FIGURE 34

A


LB
RB
AscI
I-PpoI
I-SceI
I-CeuI
Binary vector
AscI
AscI
nos promoter
nptII
nos terminator
I-PpoI
I-PpoI
assu promoter
TrBANa
tob terminator
I-SceI
I-SceI
35S² promoter
TrLARa
rbcS terminator
I-CeuI
I-CeuI
35S promoter
TrCHSa3
35S terminator
Auxiliary constructs

B

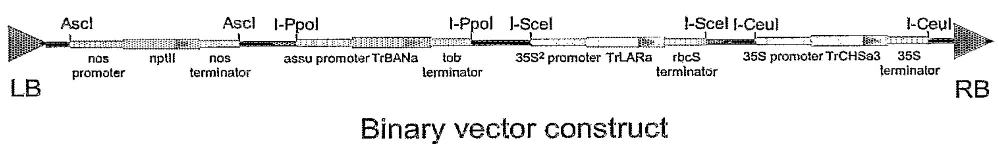

AscI
AscI
I-PpoI
I-PpoI
I-SceI
I-SceI
I-CeuI
I-CeuI
nos promoter
nptII
nos terminator
assu promoter
TrBANa
tob terminator
35S² promoter
TrLARa
rbcS terminator
35S promoter
TrCHSa3
35S terminator
LB
RB
Binary vector construct

FIGURE 35

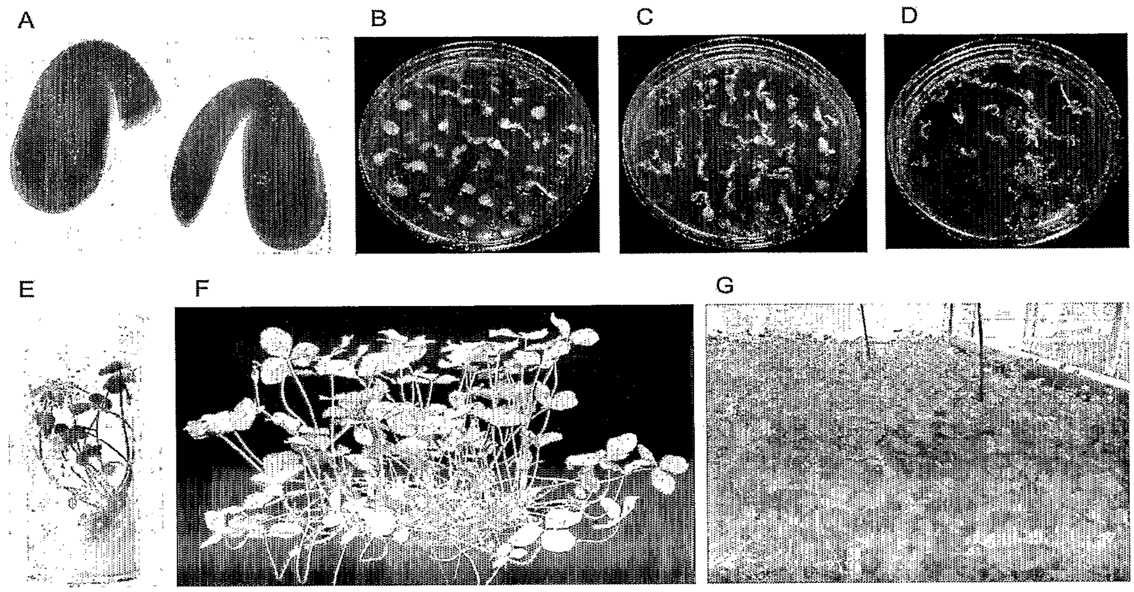


FIGURE 36

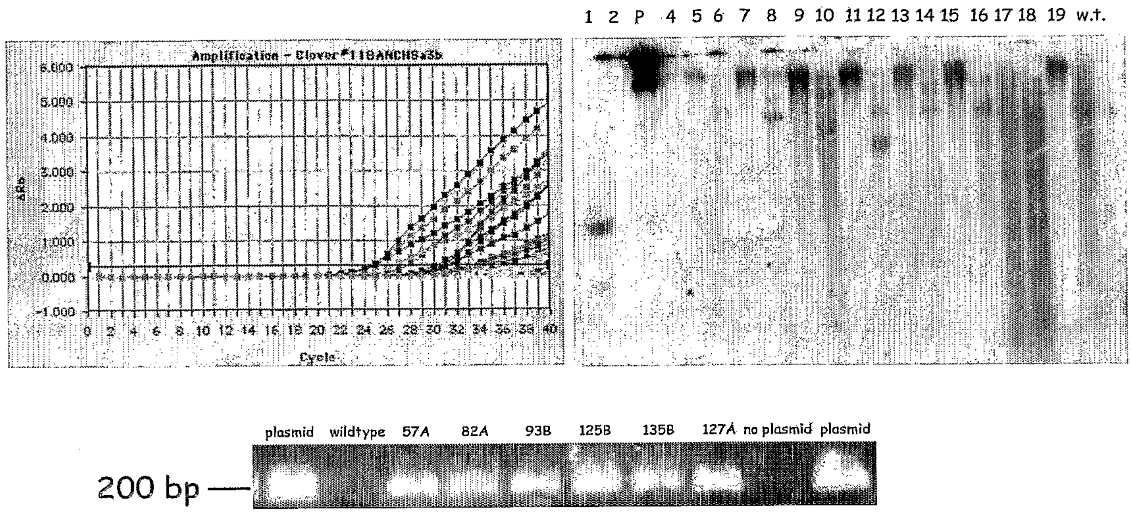
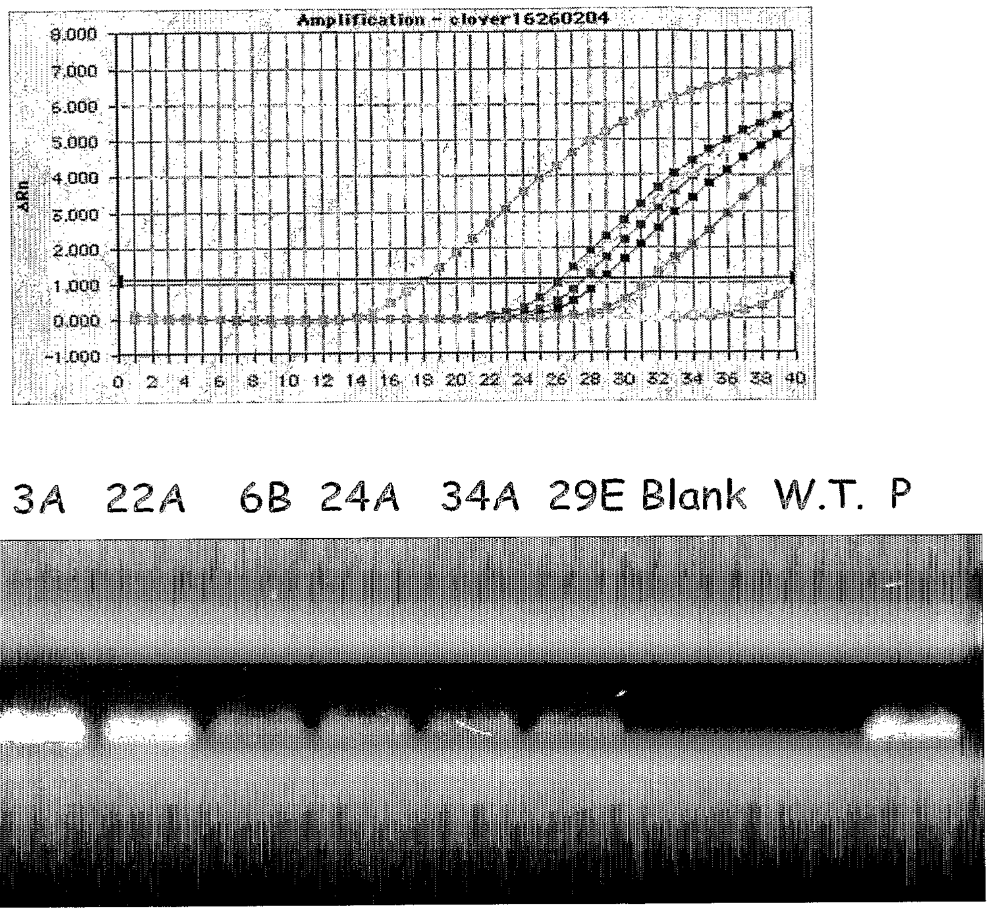

Cycle
6.000
5.000
4.000
3.000
2.000
1.000
0.000
-1.000
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
1 2 P 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 w.t.
plasmid wildtype 57A 82A 93B 125B 135B 127A no plasmid plasmid
200 bp

FIGURE 37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ΔRn
9.000
7.000
6.000
5.000
4.000
3.000
2.000
1.000
0.000
-1.000
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
1Kb+ 3A 22A 6B 24A 34A 29E Blank W.T. P

FIGURE 38

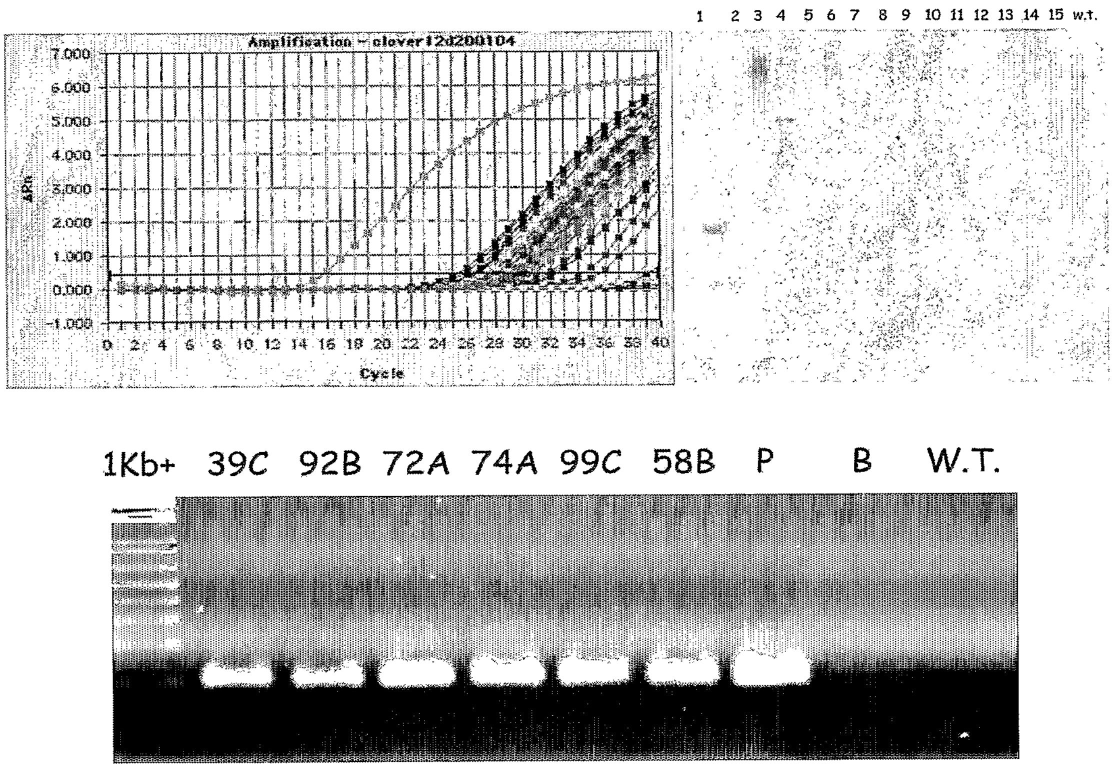
Amplification - clover12d200104
7.000
6.000
5.000
4.000
3.000
2.000
1.000
0.000
-1.000
ΔRn
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
Cycle
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 w.t.
1Kb+ 39C 92B 72A 74A 99C 58B P B W.T.

FIGURE 39

M80676490.ST25
SEQUENCE LISTING

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AgResearch Limited

<120> Manipulation of condensed tannin biosynthesis

<130> M80676490:DLT:cl

<150> 2003901797
<151> 2003-04-14

<150> 2003904369
<151> 2003-08-14

<160> 77

<170> PatentIn version 3.2

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<213> Trifolium repens

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taagatatgg tgagtgtagc tgaaattcgc aaggctcaga gggctgaagg ccctgcaacc     180

attttggcca ttggcactgc aaatccacca aaccgtgttg agcagagcac atatcctgat     240

ttctacttca aaattacaaa cagtgagcac aagactgagc tcaaagagaa gttccaacgc     300

atgtgtgaca aatccatgat caagagcaga tacatgtatc taacagaaga gattttgaaa     360

gaaaatccta gtctttgtga atacatggca ccttcattgg atgctaggca agacatggtg     420

gtggttgagg tacctagact tgggaaggag gctgcagtca aggccattaa agaatggggt     480

caaccaaagt caaagattac tcacttaatc ttttgcacca caagtggtgt tgacatgcct     540

ggtgctgatt accaactcac aaaactctta ggtcttcgcc catatgtgaa aaggtatatg     600

atgtaccaac aaggttgttt tgcaggaggc acggtgcttc gtttggcaaa agatttggcc     660

gagaacaaca aaggtgctcg tgtgctagtt gtttgttctg aagtcaccgc agtcacattt     720

cgcggcccca gtgatactca cttggacagt cttgttggac aagcattgtt tggagatgga     780

gccgctgcac taattgttgg ttctgatcca gtgcctgaaa ttgagaaacc aatatttgag     840

atggtttgga ctgcacaaac aattgctcca gacagtgaag gtgccattga tggtcatctt     900

cgtgaagctg ggctaacatt tcatcttctt aaagatgttc ctgggattgt atcaaagaac     960

attaataaag cattggttga ggctttccaa ccattaggaa tttctgacta caactcaatc    1020

ttttggattg cacacccggg tggacctgca attcttgatc aagtagaaca aaagctagcc    1080

ttgaagcccg aaaagatgag ggccacgagg gaagttctaa gtgaatatgg aaacatgtca    1140

agcgcatgtg tattgttcat cttagatgag atgcggaaga aatcggctca aaatggactt    1200

aagacaactg gagaaggact tgattggggt gtgttgttcg gcttcggacc aggacttacc    1260

attgaaaccg ttgttcttcg tagcgtggct atataagatg tgtgattgtt tttattttaa    1320
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<212> PRT
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20 25 30

Gln Ser Thr Tyr Pro Asp Phe Tyr Phe Lys Ile Thr Asn Ser Glu His
35 40 45

Lys Thr Glu Leu Lys Glu Lys Phe Gln Arg Met Cys Asp Lys Ser Met
50 55 60

Ile Lys Ser Arg Tyr Met Tyr Leu Thr Glu Glu Ile Leu Lys Glu Asn
65 70 75 80

Pro Ser Leu Cys Glu Tyr Met Ala Pro Ser Leu Asp Ala Arg Gln Asp
85 90 95

Met Val Val Val Glu Val Pro Arg Leu Gly Lys Glu Ala Ala Val Lys
100 105 110

Ala Ile Lys Glu Trp Gly Gln Pro Lys Ser Lys Ile Thr His Leu Ile
115 120 125

Phe Cys Thr Thr Ser Gly Val Asp Met Pro Gly Ala Asp Tyr Gln Leu
130 135 140

Thr Lys Leu Leu Gly Leu Arg Pro Tyr Val Lys Arg Tyr Met Met Tyr
145 150 155 160

Gln Gln Gly Cys Phe Ala Gly Gly Thr Val Leu Arg Leu Ala Lys Asp
165 170 175

Leu Ala Glu Asn Asn Lys Gly Ala Arg Val Leu Val Val Cys Ser Glu
180 185 190

Val Thr Ala Val Thr Phe Arg Gly Pro Ser Asp Thr His Leu Asp Ser
195 200 205

Leu Val Gly Gln Ala Leu Phe Gly Asp Gly Ala Ala Ala Leu Ile Val

210 215 220

Gly Ser Asp Pro Val Pro Glu Ile Glu Lys Pro Ile Phe Glu Met Val
225 230 235 240

Trp Thr Ala Gln Thr Ile Ala Pro Asp Ser Glu Gly Ala Ile Asp Gly
245 250 255

His Leu Arg Glu Ala Gly Leu Thr Phe His Leu Leu Lys Asp Val Pro
260 265 270

Gly Ile Val Ser Lys Asn Ile Asn Lys Ala Leu Val Glu Ala Phe Gln
275 280 285

Pro Leu Gly Ile Ser Asp Tyr Asn Ser Ile Phe Trp Ile Ala His Pro
290 295 300

Gly Gly Pro Ala Ile Leu Asp Gln Val Glu Gln Lys Leu Ala Leu Lys
305 310 315 320

Pro Glu Lys Met Arg Ala Thr Arg Glu Val Leu Ser Glu Tyr Gly Asn
325 330 335

Met Ser Ser Ala Cys Val Leu Phe Ile Leu Asp Glu Met Arg Lys Lys
340 345 350

Ser Ala Gln Asn Gly Leu Lys Thr Thr Gly Glu Gly Leu Asp Trp Gly
355 360 365

Val Leu Phe Gly Phe Gly Pro Gly Leu Thr Ile Glu Thr Val Val Leu
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Arg Ser Val Ala Ile
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gacgaaggta tagtgagagg tgtcacaaag cagacaaccc ctgggaaggc tactatattg 180

gctcttggca aggcattccc tcaccaactt gtgatgcaag agtgtttagt tgatggttat 240

tttagggaca ctaattgtga caatcctgaa cttaagcaga aacttgctag actttgtaag 300

acaaccacgg taaaaacaag gtatgttgtt atgaatgagg agatactaaa gaaatatcca 360

gaacttgttg tcgaaggcgc ctcaactgta aaacaacgtt tagagatatg taatgaggca 420

gtaacacaaa tggcaattga agcttcccaa gtttgcctaa agaattgggg tagatcctta 480

tcggacataa ctcatgtggt ttatgtttca tctagtgaag ctagattacc cggtggtgac 540
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tctggatgct cgggaggcgt agccggcctt cgcgttgcga aagacgtagc tgagaacaac 660
cctggaagta gagttttgct tgctacttcg gaaactacaa ttattggatt caagccacca 720
agtgttgata gaccttatga tcttgttggt gtggcactct ttggagatgg tgctggtgca 780
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aatactattg tttatgtgct ggaatatatg ctagaagagg aaaagaagat taaaaaggcg 1200
ggtggaggag attctgaatg gggattgata cttgcttttg gacctggaat tacttttgag 1260
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gtatctaaca cctgttgcaa gaaatagtaa gttattagga gatgtgcggt tacgaaattc 2040
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aaatgtaaac ttaaagagac cgaacacaca aacattgcaa ctcagatgga attgctgcca 2160
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cgccttgtct attgtagcat acaacaaacg tacagtgggt ttgtccctct caaaatggca 2280
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M80676490.ST25

<212> PRT
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35 40 45

Asp Asn Pro Glu Leu Lys Gln Lys Leu Ala Arg Leu Cys Lys Thr Thr
50 55 60

Thr Val Lys Thr Arg Tyr Val Val Met Asn Glu Glu Ile Leu Lys Lys
65 70 75 80

Tyr Pro Glu Leu Val Val Glu Gly Ala Ser Thr Val Lys Gln Arg Leu
85 90 95

Glu Ile Cys Asn Glu Ala Val Thr Gln Met Ala Ile Glu Ala Ser Gln
100 105 110

Val Cys Leu Lys Asn Trp Gly Arg Ser Leu Ser Asp Ile Thr His Val
115 120 125

Val Tyr Val Ser Ser Ser Glu Ala Arg Leu Pro Gly Gly Asp Leu Tyr
130 135 140

Leu Ser Lys Gly Leu Gly Leu Asn Pro Lys Ile Gln Arg Thr Met Leu
145 150 155 160

Tyr Phe Ser Gly Cys Ser Gly Gly Val Ala Gly Leu Arg Val Ala Lys
165 170 175

Asp Val Ala Glu Asn Asn Pro Gly Ser Arg Val Leu Leu Ala Thr Ser
180 185 190

Glu Thr Thr Ile Ile Gly Phe Lys Pro Pro Ser Val Asp Arg Pro Tyr
195 200 205

Asp Leu Val Gly Val Ala Leu Phe Gly Asp Gly Ala Gly Ala Met Ile
210 215 220

Ile Gly Ser Asp Pro Val Phe Glu Thr Glu Thr Pro Leu Phe Glu Leu
225 230 235 240

His Thr Ser Ala Gln Glu Phe Ile Pro Asp Thr Glu Lys Lys Ile Asp
245 250 255

Gly Arg Leu Thr Glu Glu Gly Ile Ser Phe Thr Leu Ala Arg Glu Leu
260 265 270

Pro Gln Ile Ile Glu Asp Asn Val Glu Gly Phe Cys Asn Lys Leu Ile
275 280 285

Asp Val Val Gly Leu Glu Asn Lys Glu Tyr Asn Lys Leu Phe Trp Ala
290 295 300

Val His Pro Gly Gly Pro Ala Ile Leu Asn Arg Val Glu Lys Arg Leu
305 310 315 320

Glu Leu Ser Pro Gln Lys Leu Asn Ala Ser Arg Lys Ala Leu Met Asp
325 330 335

Tyr Gly Asn Ala Ser Ser Asn Thr Ile Val Tyr Val Leu Glu Tyr Met
340 345 350

Leu Glu Glu Glu Lys Lys Ile Lys Lys Ala Gly Gly Gly Asp Ser Glu
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Leu Ala Arg Asn Leu Cys Ala
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gtaatgctct atttcctcgg ttgctacggc ggtgtcactg gcttacgtgt cgccaaagac 660
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20 25 30

Lys Ala Phe Pro Ala Gln Val Leu Pro Gln Glu Cys Leu Val Glu Gly
35 40 45

Phe Ile Arg Asp Thr Lys Cys Asp Asp Thr Tyr Ile Lys Glu Lys Leu
50 55 60

Glu Arg Leu Cys Lys Asn Thr Thr Val Lys Thr Arg Tyr Thr Val Met
65 70 75 80

Ser Lys Glu Ile Leu Asp Asn Tyr Pro Glu Leu Ala Ile Asp Gly Thr
85 90 95

Pro Thr Ile Arg Gln Lys Leu Glu Ile Ala Asn Pro Ala Val Val Glu
100 105 110

Met Ala Thr Arg Ala Ser Lys Asp Cys Ile Lys Glu Trp Gly Arg Ser

115 120 125

Pro Gln Asp Ile Thr His Ile Val Tyr Val Ser Ser Ser Glu Ile Arg
130 135 140

Leu Pro Gly Gly Asp Leu Tyr Leu Ala Asn Glu Leu Gly Leu Asn Ser
145 150 155 160

Asp Val Asn Arg Val Met Leu Tyr Phe Leu Gly Cys Tyr Gly Gly Val
165 170 175

Thr Gly Leu Arg Val Ala Lys Asp Ile Ala Glu Asn Asn Pro Gly Ser
180 185 190

Arg Val Leu Leu Thr Thr Ser Glu Thr Thr Ile Leu Gly Phe Arg Pro
195 200 205

Pro Ser Lys Ala Arg Pro Tyr Asp Leu Val Gly Ala Ala Leu Phe Gly
210 215 220

Asp Gly Ala Ala Ala Ala Ile Ile Gly Thr Asp Pro Ile Leu Asn Gln
225 230 235 240

Glu Ser Pro Phe Met Glu Leu Asn His Ala Val Gln Lys Phe Leu Pro
245 250 255

Asp Thr Gln Asn Val Ile Asp Gly Arg Ile Thr Glu Glu Gly Ile Asn
260 265 270

Phe Lys Leu Gly Arg Asp Leu Pro Gln Lys Ile Glu Asp Asn Ile Glu
275 280 285

Glu Phe Cys Lys Lys Ile Met Ala Lys Ser Asp Val Lys Glu Phe Asn
290 295 300

Asp Leu Phe Trp Ala Val His Pro Gly Gly Pro Ala Ile Leu Asn Lys
305 310 315 320

Leu Glu Asn Ile Leu Lys Leu Lys Ser Asp Lys Leu Asp Cys Ser Arg
325 330 335

Lys Ala Leu Met Asp Tyr Gly Asn Val Ser Ser Asn Thr Ile Phe Tyr
340 345 350

Val Met Glu Tyr Met Arg Asp Tyr Leu Lys Glu Asp Gly Ser Glu Glu
355 360 365

Trp Gly Leu Gly Leu Ala Phe Gly Pro Gly Ile Thr Phe Glu Gly Val
370 375 380

Leu Leu Arg Ser Leu

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tatcttgggt acatcttttg ttacctccaa caaaaaaatg gtgaccgtag aagagattcg 180

taacgcccaa cgttcaaatg gccctgccac tatcttagct tttggcacag ccactccttc 240

taactgtgtc actcaagctg attatcctga ttactacttt cgtatcacca acagcgaaca 300

tatgactgat cttaaggaaa aattcaagcg gatgtgtgat agatcaatga taaagaaacg 360

ttacatgcac ctaacagaag actttctgaa ggagaatcca aatatgtgtg aatacatggc 420

accatcacta gatgtaagac gagacatagt ggttgttgaa gtaccaaagc taggtaaaga 480

agcagcaaaa aaagccatat gtgaatgggg acaaccaaaa tccaaaatca cacatcttgt 540

tttctgcacc acttccggtg ttgacatgcc gggagccgat taccaactca ccaaactttt 600

aggcttaaaa ccttctgtca agcgtctcat gatgtatcaa caaggttgtt tcgctggcgg 660

cacagttctc cgcttagcaa aagaccttgt tgagaataac aaaaatgcaa gagttcttgt 720

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gctcgtggga caggcgcttt ttggtgatgg agccgcagca atgattattg gtgcggatcc 840

tgatttaacc gtggagcgtc cgattttcga gattgtttcg gctgctcaga ctattcttcc 900

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gaaagatgtt ccggggatta tttcaaagaa cattgaaaaa agtttagttg aagcttttgc 1020

gcctattggg attaatgatt ggaactcaat attttgggtt gcacatccag gtggaccggc 1080

tattttagac caggttgaag agaaactcca tcttaaagag gagaaactcc ggtccacccg 1140

gcatgtgctt agtgaatatg gaaatatgtc aagtgcatgt gttttattta ttttggatga 1200

aatgagaaag aggtctaaag aggaagggat gattacaact ggtgaagggt tggaatgggg 1260

tgtgttgttt gggtttggac cgggtttaac tgttgaaacc gttgtgcttc atagtgttcc 1320

ggttcagggt tgaatttatt atacatagat tggaaaataa aatttgcctg ccgagagatg 1380

tgaactaact ttgtaggcaa gctcaaatta aagtttgaga taatattgtg ctttagttat 1440

tatggtatgt aatgtaatgt ttttactttt ttcgaaattc atgtaatttg atatgtaaag 1500

taatatgttt gggttggaat ataattattt gttaactaaa aaaaaaaaaa aaaaaaaaaa 1560

aaaaagtact ctgcgttgtt accactgctt aatcgaattc 1600

<210> 8
<211> 391
<212> PRT
<213> Trifolium repens

M80676490.ST25

<400> 8

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20 25 30

Gln Ala Asp Tyr Pro Asp Tyr Tyr Phe Arg Ile Thr Asn Ser Glu His
35 40 45

Met Thr Asp Leu Lys Glu Lys Phe Lys Arg Met Cys Asp Arg Ser Met
50 55 60

Ile Lys Lys Arg Tyr Met His Leu Thr Glu Asp Phe Leu Lys Glu Asn
65 70 75 80

Pro Asn Met Cys Glu Tyr Met Ala Pro Ser Leu Asp Val Arg Arg Asp
85 90 95

Ile Val Val Val Glu Val Pro Lys Leu Gly Lys Glu Ala Ala Lys Lys
100 105 110

Ala Ile Cys Glu Trp Gly Gln Pro Lys Ser Lys Ile Thr His Leu Val
115 120 125

Phe Cys Thr Thr Ser Gly Val Asp Met Pro Gly Ala Asp Tyr Gln Leu
130 135 140

Thr Lys Leu Leu Gly Leu Lys Pro Ser Val Lys Arg Leu Met Met Tyr
145 150 155 160

Gln Gln Gly Cys Phe Ala Gly Gly Thr Val Leu Arg Leu Ala Lys Asp
165 170 175

Leu Val Glu Asn Asn Lys Asn Ala Arg Val Leu Val Val Cys Ser Glu
180 185 190

Ile Thr Ala Val Thr Phe Arg Gly Pro Ser Asp Thr His Leu Asp Ser
195 200 205

Leu Val Gly Gln Ala Leu Phe Gly Asp Gly Ala Ala Ala Met Ile Ile
210 215 220

Gly Ala Asp Pro Asp Leu Thr Val Glu Arg Pro Ile Phe Glu Ile Val
225 230 235 240

Ser Ala Ala Gln Thr Ile Leu Pro Asp Ser Asp Gly Ala Ile Asp Gly
245 250 255

His Leu Arg Glu Val Gly Leu Thr Phe His Leu Leu Lys Asp Val Pro

260 265 270

Gly Ile Ile Ser Lys Asn Ile Glu Lys Ser Leu Val Glu Ala Phe Ala
275 280 285

Pro Ile Gly Ile Asn Asp Trp Asn Ser Ile Phe Trp Val Ala His Pro
290 295 300

Gly Gly Pro Ala Ile Leu Asp Gln Val Glu Glu Lys Leu His Leu Lys
305 310 315 320

Glu Glu Lys Leu Arg Ser Thr Arg His Val Leu Ser Glu Tyr Gly Asn
325 330 335

Met Ser Ser Ala Cys Val Leu Phe Ile Leu Asp Glu Met Arg Lys Arg
340 345 350

Ser Lys Glu Glu Gly Met Ile Thr Thr Gly Glu Gly Leu Glu Trp Gly
355 360 365

Val Leu Phe Gly Phe Gly Pro Gly Leu Thr Val Glu Thr Val Val Leu
370 375 380

His Ser Val Pro Val Gln Gly
385 390

<210> 9
<211> 1309
<212> DNA
<213> Trifolium repens

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ttggaaacaa gaaagcatgt gtgattggtg gcactggttt tgttgcatct atgttgatca 180
agcagttact tgaaaagggt tatgctgtta atactaccgt tagagaccca gatagcccta 240
agaaaatatc tcacctagtg gcactgcaaa gtttggggga actgaatcta tttagagcag 300
acttaacagt tgaagaagat tttgatgctc ctatagcagg atgtgaactt gtttttcaac 360
ttgctacacc tgtgaacttt gcttctcaag atcctgagaa tgacatgata aagccagcaa 420
tcaaaggtgt gttgaatgtg ttgaaagcaa ttgcaagagc aaaagaagtt aaaagagtta 480
tcttaacatc ttcggcagcc gcggtgacta taaatgaact caaagggaca ggtcatgtta 540
tggatgaaac caactggtct gatgttgaat ttctcaacac tgcaaaacca cccacttggg 600
gttatcctgc ctcaaaaatg ctagctgaaa aggctgcatg gaaatttgct gaagaaaatg 660
acattgatct aatcactgtg atacctagtt taacaactgg tccttctctc acaccagata 720
tcccatctag tgttggcttg gcaatgtctc taataacagg caatgatttt ctcataaatg 780
ctttgaaagg aatgcagttt ctgtcgggtt cgttatccat cactcatgtt gaggatattt 840

gccgagctca tatatttctt gcagagaaag aatcagcttc tggtagatac atttgctgtg 900

ctcacaatac tagtgttccc gagcttgcaa agtttctcaa caaacgatat cctcagtata 960

aagttccaac tgaatttgat gattgcccca gcaaggcaaa gttgataatc tcttctgaaa 1020

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tcgagtattt taagactaag gggcactga agaattagat tttgatattt ctaattcaat 1140

agcaaactct aagcttgtta tgtgtttgtg aagttcagag tgaaatatca aatgaataag 1200

tggagagagc acaataagag gagagcacaa taattttgga aaaaaaaaaa aaaaaaaaaa 1260

aaaaaaaagt actctgcgtt gttaccactg cttaatcact agtgaattc 1309

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<211> 338
<212> PRT
<213> Trifolium repens

<400> 10

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Gly Thr Gly Phe Val Ala Ser Met Leu Ile Lys Gln Leu Leu Glu Lys
20 25 30

Gly Tyr Ala Val Asn Thr Thr Val Arg Asp Pro Asp Ser Pro Lys Lys
35 40 45

Ile Ser His Leu Val Ala Leu Gln Ser Leu Gly Glu Leu Asn Leu Phe
50 55 60

Arg Ala Asp Leu Thr Val Glu Glu Asp Phe Asp Ala Pro Ile Ala Gly
65 70 75 80

Cys Glu Leu Val Phe Gln Leu Ala Thr Pro Val Asn Phe Ala Ser Gln
85 90 95

Asp Pro Glu Asn Asp Met Ile Lys Pro Ala Ile Lys Gly Val Leu Asn
100 105 110

Val Leu Lys Ala Ile Ala Arg Ala Lys Glu Val Lys Arg Val Ile Leu
115 120 125

Thr Ser Ser Ala Ala Ala Val Thr Ile Asn Glu Leu Lys Gly Thr Gly
130 135 140

His Val Met Asp Glu Thr Asn Trp Ser Asp Val Glu Phe Leu Asn Thr
145 150 155 160

Ala Lys Pro Pro Thr Trp Gly Tyr Pro Ala Ser Lys Met Leu Ala Glu
165 170 175

Lys Ala Ala Trp Lys Phe Ala Glu Glu Asn Asp Ile Asp Leu Ile Thr
180 185 190

Val Ile Pro Ser Leu Thr Thr Gly Pro Ser Leu Thr Pro Asp Ile Pro
195 200 205

Ser Ser Val Gly Leu Ala Met Ser Leu Ile Thr Gly Asn Asp Phe Leu
210 215 220

Ile Asn Ala Leu Lys Gly Met Gln Phe Leu Ser Gly Ser Leu Ser Ile
225 230 235 240

Thr His Val Glu Asp Ile Cys Arg Ala His Ile Phe Leu Ala Glu Lys
245 250 255

Glu Ser Ala Ser Gly Arg Tyr Ile Cys Cys Ala His Asn Thr Ser Val
260 265 270

Pro Glu Leu Ala Lys Phe Leu Asn Lys Arg Tyr Pro Gln Tyr Lys Val
275 280 285

Pro Thr Glu Phe Asp Asp Cys Pro Ser Lys Ala Lys Leu Ile Ile Ser
290 295 300

Ser Glu Lys Leu Ile Lys Glu Gly Phe Ser Phe Lys His Gly Ile Ala
305 310 315 320

Glu Thr Phe Asp Gln Thr Val Glu Tyr Phe Lys Thr Lys Gly Ala Leu
325 330 335

Lys Asn

<210> 11
<211> 1409
<212> DNA
<213> Trifolium repens

<400> 11
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taactctaaa agcaaagcaa tggcaccagc agcaacatca tcaccaacca ctcctactac 120
taccaagggt cgtgtcctaa ttgttggagg aacaggtttc attggaaaat ttgtaactga 180
ggcaagtctt tccacaacac acccaaccta cttgttggtt cggccaggac ctcttctctc 240
ttctaaggct gccactatta aggcattcca agagaaaggt gccattgtca tttatggtcg 300
ggtaaataat aaggagttca tggagatgat tttgaaaaag tatgagataa atgtagtcat 360
ttctgcaata ggaggctctg atggcttgct ggaacagctt actttggtgg aggccatgaa 420
atctattaac accattaaga ggtttttgcc ttcggaattt ggtcacgatg tggacagagc 480
aaatcctgtg gaacctggcc taacaatgta caaacagaaa cgtttggtta gacgtgtgat 540

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cgaagaatct ggtataccat acacctacat ctgttgcaat tcgatcgcat cttggccgta     600
ctatgacaat tgtcatccat cacagcttcc tccaccgttg gatcaattac atatttatgg     660
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ggtcattgat gatgaaagaa caatcaacaa aaatgttcat tttcgacctt ctaacaattg     780
ttatagcatg aatgagcttg cttctttgtg ggaaaacaaa attgcacgaa aaattcctag     840
agtgatcgtc tctgaagacg atcttctagc aatagccgca gaaaattgca taccggaaag     900
tgtcgtggca ccaatcactc atgatatatt catcaatgga tgtcaagtta acttcaagat     960
agatggaatt catgatgttg aaattggcac tctatatcct ggtgaatcgg taagaagttt    1020
ggaggaatgc tatgagaaat ttgttgtcat ggcggctgac aagattcata aagaagaaac    1080
tggagttacc gcaggtgggg gcggcacaac ggctatggta gagccggtgc caatcacagc    1140
ttcctgttga aaaggttcac ctgaggtgga tattcttttg agtcataaga catgttgatt    1200
gttgatgttg ttttcaagaa tgtttcatca tttcatgtgt tttattaatc ctaagtacaa    1260
ataattgctg tctacgtacg ttcttagttg caaaaattct tgttattctc tattgaggta    1320
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<210>  12
<211>  356
<212>  PRT
<213>  Trifolium repens

<400>  12

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Gly Arg Val Leu Ile Val Gly Gly Thr Gly Phe Ile Gly Lys Phe Val
            20                  25                  30

Thr Glu Ala Ser Leu Ser Thr Thr His Pro Thr Tyr Leu Leu Val Arg
        35                  40                  45

Pro Gly Pro Leu Leu Ser Ser Lys Ala Ala Thr Ile Lys Ala Phe Gln
    50                  55                  60

Glu Lys Gly Ala Ile Val Ile Tyr Gly Arg Val Asn Asn Lys Glu Phe
65                  70                  75                  80

Met Glu Met Ile Leu Lys Lys Tyr Glu Ile Asn Val Val Ile Ser Ala
                85                  90                  95

Ile Gly Gly Ser Asp Gly Leu Leu Glu Gln Leu Thr Leu Val Glu Ala
            100                 105                 110

Met Lys Ser Ile Asn Thr Ile Lys Arg Phe Leu Pro Ser Glu Phe Gly
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115 120 125

His Asp Val Asp Arg Ala Asn Pro Val Glu Pro Gly Leu Thr Met Tyr
130 135 140

Lys Gln Lys Arg Leu Val Arg Arg Val Ile Glu Glu Ser Gly Ile Pro
145 150 155 160

Tyr Thr Tyr Ile Cys Cys Asn Ser Ile Ala Ser Trp Pro Tyr Tyr Asp
165 170 175

Asn Cys His Pro Ser Gln Leu Pro Pro Pro Leu Asp Gln Leu His Ile
180 185 190

Tyr Gly His Gly Asp Val Lys Ala Tyr Phe Val Asp Gly Tyr Asp Ile
195 200 205

Gly Lys Phe Thr Met Lys Val Ile Asp Asp Glu Arg Thr Ile Asn Lys
210 215 220

Asn Val His Phe Arg Pro Ser Asn Asn Cys Tyr Ser Met Asn Glu Leu
225 230 235 240

Ala Ser Leu Trp Glu Asn Lys Ile Ala Arg Lys Ile Pro Arg Val Ile
245 250 255

Val Ser Glu Asp Asp Leu Leu Ala Ile Ala Ala Glu Asn Cys Ile Pro
260 265 270

Glu Ser Val Val Ala Pro Ile Thr His Asp Ile Phe Ile Asn Gly Cys
275 280 285

Gln Val Asn Phe Lys Ile Asp Gly Ile His Asp Val Glu Ile Gly Thr
290 295 300

Leu Tyr Pro Gly Glu Ser Val Arg Ser Leu Glu Glu Cys Tyr Glu Lys
305 310 315 320

Phe Val Val Met Ala Ala Asp Lys Ile His Lys Glu Glu Thr Gly Val
325 330 335

Thr Ala Gly Gly Gly Gly Thr Thr Ala Met Val Glu Pro Val Pro Ile
340 345 350

Thr Ala Ser Cys
355

<210> 13
<211> 1551
<212> DNA
<213> Trifolium repens

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accaacattg tcacaattaa ctctaaaagc aaagcaatgg caccagcagc aacatcatca 120
ccaaccactc ctactactac caagggtcgt gtcctaattg ttggaggaac aggtttcatt 180
ggaaaatttg taactgaggc aagtctttcc acaacacacc caacctactt gttggttcgg 240
ccaggacctc ttctctcttc taaggctgcc actattaagg cattccaaga gaaaggtgcc 300
attgtcattt atggtcgggt aaataataag gagttcatgg agatgatttt gaaaaagtat 360
gagataaatg tagtcatttc tgcaatagga ggctctgatg gcttgctgga acagcttact 420
ttggtggagg ccatgaaatc tattaacacc attaagaggt ttttgccttc agaatttggt 480
cacgatgtgg acagagcaaa tcctgtggaa cctggcctaa caatgtacaa acagaaacgt 540
ttggttagac gtgtgatcga agaatctggt gtaccataca cctacatctg ttgcaattcg 600
atcgcatcct ggccgtacta tgacaattgt catccatcac agcttcctcc accgttggat 660
caattacata tttatggtca tggcgatgtc aaagcttact ttgttgatgg ctatgatatt 720
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cgaccttcta acaattgtta tagcatgaat gagcttgctt ctttgtggga aaacaaaatt 840
gcacgaaaaa ttcctagagt gatcgtctct gaagacgatc ttctagcaat agccgcagaa 900
aactgcatac cggaaagtgt tgtggcatca atcactcatg atatattcat caatggatgt 960
caagttaact tcaaggtaga tggaattcat gatgttgaaa ttggcactct atatcctggt 1020
gaatcggtaa gaagtttgga ggaatgctat gagaaatttg ttgtcatggc ggctgacaag 1080
attcataaag aagaaactgg agttaccgca ggtgggggcg gcacaacggc tatggtagag 1140
ccggtgccaa tcacagcttc ctgttgaaaa ggttcacctg aggtggatat tcttttgagt 1200
cataagacat gttgattgtt gatgttgttt tcaagaatgt ttcatcattt catgtgtttt 1260
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gctatttgca ataatgattt ttgtgaagca cttgtggtgt atttacttac tactgaaaat 1440
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aaaaaaaaaa gtactctgcg ttgttaccac tgcttaatca ctagtgaatt c 1551

<210> 14
<211> 356
<212> PRT
<213> Trifolium repens

<400> 14

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Gly Arg Val Leu Ile Val Gly Gly Thr Gly Phe Ile Gly Lys Phe Val
20 25 30

Thr Glu Ala Ser Leu Ser Thr Thr His Pro Thr Tyr Leu Leu Val Arg
35 40 45

Pro Gly Pro Leu Leu Ser Ser Lys Ala Ala Thr Ile Lys Ala Phe Gln
50 55 60

Glu Lys Gly Ala Ile Val Ile Tyr Gly Arg Val Asn Asn Lys Glu Phe
65 70 75 80

Met Glu Met Ile Leu Lys Lys Tyr Glu Ile Asn Val Val Ile Ser Ala
85 90 95

Ile Gly Gly Ser Asp Gly Leu Leu Glu Gln Leu Thr Leu Val Glu Ala
100 105 110

Met Lys Ser Ile Asn Thr Ile Lys Arg Phe Leu Pro Ser Glu Phe Gly
115 120 125

His Asp Val Asp Arg Ala Asn Pro Val Glu Pro Gly Leu Thr Met Tyr
130 135 140

Lys Gln Lys Arg Leu Val Arg Arg Val Ile Glu Glu Ser Gly Val Pro
145 150 155 160

Tyr Thr Tyr Ile Cys Cys Asn Ser Ile Ala Ser Trp Pro Tyr Tyr Asp
165 170 175

Asn Cys His Pro Ser Gln Leu Pro Pro Pro Leu Asp Gln Leu His Ile
180 185 190

Tyr Gly His Gly Asp Val Lys Ala Tyr Phe Val Asp Gly Tyr Asp Ile
195 200 205

Gly Lys Phe Thr Met Lys Val Ile Asp Asp Glu Arg Thr Ile Asn Lys
210 215 220

Asn Val His Phe Arg Pro Ser Asn Asn Cys Tyr Ser Met Asn Glu Leu
225 230 235 240

Ala Ser Leu Trp Glu Asn Lys Ile Ala Arg Lys Ile Pro Arg Val Ile
245 250 255

Val Ser Glu Asp Asp Leu Leu Ala Ile Ala Ala Glu Asn Cys Ile Pro
260 265 270

Glu Ser Val Val Ala Ser Ile Thr His Asp Ile Phe Ile Asn Gly Cys
275 280 285

Gln Val Asn Phe Lys Val Asp Gly Ile His Asp Val Glu Ile Gly Thr
290 295 300

Leu Tyr Pro Gly Glu Ser Val Arg Ser Leu Glu Glu Cys Tyr Glu Lys
305 310 315 320

Phe Val Val Met Ala Ala Asp Lys Ile His Lys Glu Glu Thr Gly Val
325 330 335

Thr Ala Gly Gly Gly Gly Thr Thr Ala Met Val Glu Pro Val Pro Ile
340 345 350

Thr Ala Ser Cys
355

<210> 15
<211> 1384
<212> DNA
<213> Trifolium repens

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taactctaaa agtaaagcaa tggcaccagc agcaacatca tcaccaacca ctcccactac 120

taccaagggt cgtgtcctaa ttgttggagg aacaggtttc attggaaaat ttgtaactga 180

ggcaagtctt tccacaacac acccaaccta cttgttggtt cggccaggac ctcttctctc 240

ttctaaggct gccactatta aggcattcca agagaaaggt gccattgtca tttatggtcg 300

ggtaaataat aaggagttca tggagatgat tttgaaaaag tatgagataa atgtagtcat 360

ttctgcaata ggaggctctg atggcttgct ggaacagctt actttggtgg aggccatgaa 420

atctattaac accattaaga ggtttttgcc ttcggaattt ggtcacgatg tggacagagc 480

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cgaagaatct ggtataccat acacctacat ctgttgcaat tcgatcgcat cttggccgta 600

ctatgacaat tgtcatccat cacagcttcc tccaccgttg gatcaattac atatttatgg 660

tcatggcgat gtcaaagctt actttgttga tggctatgat attgggaaat tcacaatgaa 720

ggtcattgat gatgaaagaa caatcaacaa aaatgttcat tttcgacctt ctaacaattg 780

ttatagcatg aatgagcttg cttctttgtg ggaaaacaaa attgcacgaa aaattcctag 840

agtgatcgtc tctgaagacg atcttctagc aatagccgca gaaaattgca taccggaaag 900

tgtcgtggca ccaatcactc atgatatatt catcaatgga tgtcaagtta acttcaagat 960

agatggaatt catgatgttg aaattggcac tctatatcct ggtgaatcgg taagaagttt 1020

ggaggaatgc tatgagaaat ttgttgtcat ggcggctgac aagattcata aagaagaaac 1080

tggagttacc gcaggtgggg gcggcacaac ggctatggta gagccggtgc caatcacagc 1140

ttcctgttga aaaggttcac ctgaggtgga tattcttttg agtcataaga catgttgatt 1200

gttgatgttg ttttcaagaa tgtttcatca tttcatgtgt tttattaatc ctaagtacaa 1260

ataattgctg tctacgtacg ttcttagttg caaaaattct tgttattctc tatcaaaaaa 1320

aaaaaaaaaa aaaaaaaaaa aaagtactct gcgttgttac cactgcttaa tcactagtga 1380

attc 1384

<210> 16
<211> 356
<212> PRT
<213> Trifolium repens

<400> 16

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Gly Arg Val Leu Ile Val Gly Gly Thr Gly Phe Ile Gly Lys Phe Val
20 25 30

Thr Glu Ala Ser Leu Ser Thr Thr His Pro Thr Tyr Leu Leu Val Arg
35 40 45

Pro Gly Pro Leu Leu Ser Ser Lys Ala Ala Thr Ile Lys Ala Phe Gln
50 55 60

Glu Lys Gly Ala Ile Val Ile Tyr Gly Arg Val Asn Asn Lys Glu Phe
65 70 75 80

Met Glu Met Ile Leu Lys Lys Tyr Glu Ile Asn Val Val Ile Ser Ala
85 90 95

Ile Gly Gly Ser Asp Gly Leu Leu Glu Gln Leu Thr Leu Val Glu Ala
100 105 110

Met Lys Ser Ile Asn Thr Ile Lys Arg Phe Leu Pro Ser Glu Phe Gly
115 120 125

His Asp Val Asp Arg Ala Asp Pro Val Glu Pro Gly Leu Thr Met Tyr
130 135 140

Lys Gln Lys Arg Leu Val Arg Arg Val Ile Glu Glu Ser Gly Ile Pro
145 150 155 160

Tyr Thr Tyr Ile Cys Cys Asn Ser Ile Ala Ser Trp Pro Tyr Tyr Asp
165 170 175

Asn Cys His Pro Ser Gln Leu Pro Pro Pro Leu Asp Gln Leu His Ile
180 185 190

Tyr Gly His Gly Asp Val Lys Ala Tyr Phe Val Asp Gly Tyr Asp Ile
195 200 205

Gly Lys Phe Thr Met Lys Val Ile Asp Asp Glu Arg Thr Ile Asn Lys
210 215 220

Asn Val His Phe Arg Pro Ser Asn Asn Cys Tyr Ser Met Asn Glu Leu

225 230 235 240

Ala Ser Leu Trp Glu Asn Lys Ile Ala Arg Lys Ile Pro Arg Val Ile
245 250 255

Val Ser Glu Asp Asp Leu Leu Ala Ile Ala Ala Glu Asn Cys Ile Pro
260 265 270

Glu Ser Val Val Ala Pro Ile Thr His Asp Ile Phe Ile Asn Gly Cys
275 280 285

Gln Val Asn Phe Lys Ile Asp Gly Ile His Asp Val Glu Ile Gly Thr
290 295 300

Leu Tyr Pro Gly Glu Ser Val Arg Ser Leu Glu Glu Cys Tyr Glu Lys
305 310 315 320

Phe Val Val Met Ala Ala Asp Lys Ile His Lys Glu Glu Thr Gly Val
325 330 335

Thr Ala Gly Gly Gly Gly Thr Thr Ala Met Val Glu Pro Val Pro Ile
340 345 350

Thr Ala Ser Cys
355

<210> 17
<211> 18
<212> DNA
<213> Artificial

<220>
<223> Primer sequence

<400> 17
aggaggctgc agtcaagg 18

<210> 18
<211> 19
<212> DNA
<213> Artificial

<220>
<223> Primer sequence

<400> 18
tgcctgaaat tgagaaacc 19

<210> 19
<211> 18
<212> DNA
<213> Artificial

<220>
<223> Primer sequence

<400> 19

aaagctagcc ttgaagcc 18

<210> 20
<211> 19
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<220>
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<400> 20
tcggacataa ctcatgtgg 19

<210> 21
<211> 18
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<220>
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<400> 21
ttgggttgga gaataagg 18

<210> 22
<211> 18
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<220>
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<400> 22
tggacattta ttggttgc 18

<210> 23
<211> 18
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<220>
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<400> 23
tatcatgtct ggaaatgc 18

<210> 24
<211> 19
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<400> 24
agattgcatc aaagaatgg 19

<210> 25
<211> 17
<212> DNA
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<220>
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<400> 25
ggtccaaaag ccaatcc 17

<210> 26
<211> 18
<212> DNA
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<220>
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<400> 26
taagacgaga catagtgg 18

<210> 27
<211> 18
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<400> 27
tattcactaa gcacatgc 18

<210> 28
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<220>
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<400> 28
tcatttctgc aataggagg 19

<210> 29
<211> 18
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<220>
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<400> 29
atccacctca ggtgaacc 18

<210> 30
<211> 18
<212> DNA
<213> Artificial

<220>
<223> Primer sequence

<400> 30
aataggaggc tctgatgg 18

<210> 31

<211> 18
<212> DNA
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<220>
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<400> 31
atccacctca ggtgaacc 18

<210> 32
<211> 17
<212> DNA
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<220>
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<400> 32
aggctctgat ggcttgc 17

<210> 33
<211> 18
<212> DNA
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<220>
<223> Primer sequence

<400> 33
atccacctca ggtgaacc 18

<210> 34
<211> 30
<212> DNA
<213> Artificial

<220>
<223> Primer sequence

<400> 34
gaattctaga agatatggtg agtgtagctg 30

<210> 35
<211> 30
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2004/000494

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. ^{7}: C12N 15/29

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
SEE ELECTRONIC DATABASES BELOW

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
SEE ELECTRONIC DATABASES BELOW

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
DGENE, Genebank, EMBL, SwissProt, PIR, PubMed : SEQ ID 2, 4, 6, 8, 10, 12, 14, 16 ; Keywords: chalcone synthase, leucoanthocyanidin reductase, lcr, ban, dihydroflavonol 4-reductase, clover, trifolium.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	WO, A, 2003031622 (AGRICULTURE VICTORIA SERVICES PTY LTD ET.AL.) 17 April 2003 (SEQ ID 2 has 100% identity with Figure 143, SEQ ID 4 has 96% identity with Fig. 148, SEQ ID 6 has 93% identity with Figure 158, SEQ ID 8 has 94% identity with Figure 163, SEQ ID 10 has 100% identity with Figure 118,)	1-31
X	WO, A, 2002057418 (THE SALK INSTITUTE FOR BIOLOGICAL STUDIES) 25 July 2002 (SEQ ID 2 has 92% identity with SEQ ID 1 on page 13)	1-3,6-8,10-17,19-23,25-29
X	SWISS-PROT database Accession Number P51088, Chalcone synthase 6. Howles, P. A. et al. 1 October 1996 (98% identity with SEQ ID 2)	1-3,6-8,10-17,19-23,25-29
X	SWISS-PROT database Accession Number P51083, Chalcone synthase 1. Arioli, T., et. al. 1 October 1996 (97% identity with SEQ ID 2)	1-3,6-8,10-17,19-23,25-29

[X] Further documents are listed in the continuation of Box C   [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 June 2004	8 JUL 2004

Name and mailing address of the ISA/AU	Authorized officer
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	**ALISTAIR BESTOW** Telephone No : (02) 6283 2450

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GenPept database Accession Number CAA10131 Chalcone synthase, Dopico, B., et. al. 11 November 1998 (97% identity with SEQ ID 2)	1-3,6-8,10-17,19-23,25-29
X	SWISS-PROT database Accession Number Q9SEVO Leucoanthocyanidin reductase, Devic, M., et. al. 28 February 2003 (61% identity with SEQ ID 10)	1,2,4,6,7,9-16,18-22,24-28,30
X	GenPept database Accession Number AAF23859 11 January 2000 (61% identity with SEQ ID 10)	1,2,4,6,7,9-16,18-22,24-28,30
X	JEZ, J. M. et.al. Structure and Mechanism of Chalcone Synthase-like Polyketide Synthases, *Journal of Industrial Microbiology & Biotechnology* (2001) 27:393-8	1-3,6-8,10-17,19-23,25-29
X	ARIOLI, T., et.al. In *Trifolium subterraneum*, chalcone synthase is encoded by a multigene family. Gene, 138:79-86 (1994) (SEQ ID 2 is 97% identity to seq. of Fig. 3)	1-3,6-8,10-17,19-23,25-29
X	CHARRIER, B., et.al. Molecular characterization and expression of alfalfa (*Medicago sativa* L.) flavanone-3-hydroxylase and dihydroflavonol-4-reductase encoding genes. Plant Molecular Biology 1995 Nov;29(4):773-86 (See Figure 3)	1,2,4,6,7,9-16,18-22,24-28,30
X	WO A1 2002066625 (COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION ET. AL.) 29 August 2002 (SEQ ID 12 and 14 and 16 have 72% identity to SEQ ID 28)	1,2,5,6,8-15,17-21,23-28,31.
X	WO A1 2000022099 (GENESIS RESEARCH AND DEVELOPMENT CORPORATION LIMITED et. al.) 20 April 2000, (SEQ ID 12 and 14 have 56% identity to SEQ ID 323. SEQ ID 16 has 57% identity to SEQ ID 323)	1,2,5,6,8-15,17-21,23-28,31
X	EP A 1033405 (CERES INC.) 6 September 2000. (SEQ ID 4 has 77% identity with SEQ ID 66257 and SEQ ID 15839, SEQ ID 6 has 67% identity with SEQ ID 7420 and 7419)	1-3,6-8,10-17,19-23,25-29
X	WO A 2002010210 (BAYER AG) 7 February 2002. (SEQ ID 4 has 77% identity with SEQ ID 2451, SEQ ID 6 has 70% identity with SEQ ID 18.)	1-3,6-8,10-17,19-23,25-29
X	EP A 1254960 (UNILEVER PLC) 6 November 2002. (SEQ ID 8 has 81% identity with Fig.11.)	1-3,6-8,10-17,19-23,25-29
P,X	WO A 2003040306 (GENESIS RESEARCH AND & DEVELOPMENT CORPORATION LTD) 15 May 2003. (SEQ ID 8 has 78% identity with SEQ ID 119 and SEQ ID 186.)	1-3,6-8,10-17,19-23,25-29
P,X	WO A 2004020637 (INTERNATIONAL FLOWER DEVELOPMENTS PTY. LTD.) 19 March 2004. (SEQ ID 8 has 80% identity with disclosure on p.244-6.)	1-3,6-8,10-17,19-23,25-29
X	PIR database Accession Number T10231 Anther-specific protein homolog T11I11.90, Bevan, M., et. al. 16 July 1999. (77% identity with SEQ ID 4.)	1-3,6-8,10-17,19-23,25-29

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PIR database Accession Number T15054. Anther-specific protein - wood tobacco. Atanassov, I.I., et. al. 20 September 1999. (76% identity with SEQ ID 6.)	1-3,6-8,10-17,19-23,25-29
X	EMBL database Accession Number Y14507, Nicotiana sylvestris CHSLK gene. Atanassov I.I., et. al. 11 August 1997. (70% identity with SEQ ID 6.)	1-3,6-8,10-17,19-23,25-29
X	SWISS-PROT database Accession Number P51075, Chalcone synthase. Pellinen, R., et. al. 1 October 1996. (82% identity with SEQ ID 8.)	1-3,6-8,10-17,19-23,25-29
X	SWISS-PROT database Accession Number Q9FSB7, Chalcone synthase 3. Springob, K., et. al. (82% identity with SEQ ID 8), and, SPRINGOB, K., et. al. Specificities of functionally expressed chalcone and acridone synthases from *Ruta graveolens*, Eur. J. Biochem. 267(22):6552-9 (2000)	1-3,6-8,10-17,19-23,25-29
X	TREMBL database Accesssion Number BAB84112. Chalcone synthase (Vitis vinifera). Goto-Yamamoto, N., et. al. 19 July 2002. (82% identity with SEQ ID 8.)	1-3,6-8,10-17,19-23,25-29

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2004/000494

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. type of material

      - [X] a sequence listing
      - [ ] table(s) related to the sequence listing

   b. format of material

      - [ ] in written format
      - [X] in computer readable form

   c. time of filing/furnishing

      - [X] contained in the international application as filed
      - [ ] filed together with the international application in computer readable form
      - [ ] furnished subsequently to this Authority for the purposes of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table(s) relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

The Applicant has claimed more than one invention. Rule 13.1 of the PCT states the principle that an International Application should relate to only one invention or, if there is more than one invention, that the inclusion of those inventions in one International Application is only permitted if all inventions are so linked as to form a single general inventive concept.

(continued on Extra Sheet)

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

Supplemental Box
(To be used when the space in any of Boxes I to VIII is not sufficient)

Continuation of Box No: III

Rule 13.2 of the PCT defines the method for determining whether the requirement of unity of invention is satisfied in respect of a group of inventions claimed in an International application. Unity of invention exists only when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding "special technical features." The expression "special technical features" is defined in Rule 13.2 as meaning those technical features that define a contribution which each of the inventions, considered as a whole, makes over the prior art. The determination is made on the contents of the claims as interpreted in light of the description and drawings (if any).

Invention 1 : Polypeptide and polynucleotide encoding chalcone synthase of the formula TrCHSa3, represented by SEQ ID NOS 1 and 2. This invention is recited in claims 1 - 3, 6 - 8, 10 - 17, 19 - 23, 25 - 29 (in part).

Invention 2 : Polypeptide and polynucleotide encoding chalcone synthase of the formula TrCHSc, represented by SEQ ID NOS 3 and 4. This invention is recited in claims 1 - 3, 6 - 8, 10 - 17, 19 - 23, 25 - 29 (in part).

Invention 3 : Polypeptide and polynucleotide encoding chalcone synthase of the formula TrCHSf, represented by SEQ ID NOS 5 and 6. This invention is recited in claims 1 - 3, 6 - 8, 10 - 17, 19 - 23, 25 - 29 (in part).

Invention 4 : Polypeptide and polynucleotide encoding chalcone synthase of the formula TrCHSh, represented by SEQ ID NOS 7 and 8. This invention is recited in claims 1 - 3, 6 - 8, 10 - 17, 19 - 23, 25 - 29 (in part).

Invention 5 : Polypeptide and polynucleotide encoding dihydroflavonol 4-reductase of the formula TrBANa, represented by SEQ ID NOS 9 and 10. This invention is recited in claims 1, 2, 4, 6 - 7, 9, 10 - 16, 18 - 22, 24 - 30. (in part)

Invention 6 : Polypeptide and polynucleotide encoding leucoanthrocyanine reductase of the formula TrLARa, represented by SEQ ID NOS 11 and 12. This invention is recited in claims 1, 2, 5, 6, 8 - 15, 17 - 21, 23 - 26, 28, 29, 30. (in part).

Invention 7 : Polypeptide and polynucleotide encoding leucoanthrocyanine reductase of the formula TrLARb, represented by SEQ ID NOS 13 and 14. This invention is recited in claims 1, 2, 5, 6, 8 - 15, 17 - 21, 23 - 26, 28, 29, 30. (in part).

Invention 8 : Polypeptide and polynucleotide encoding leucoanthrocyanine reductase of the formula TrLARc, represented by SEQ ID NOS 15 and 16. This invention is recited in claims 1, 2, 5, 6, 8 - 15, 17 - 21, 23 - 26, 28, 29, 30. (in part).

Each of the above enzymes may be involved in flavonoid biosynthesis in plants, and more specifically the modification of the content of condensed tannins, but this not novel, as noted in the following abstract. Therefore this cannot be used as a special technical feature providing unity to all of the sequences.

XIE DY et. al. Science (Jan 2003)299(5605):396-9 Role of anthocyanin reductase, encoded by BANYULS in plant flavonoid biosynthesis.

Although some of the claims share a technical feature whereby they are a chalcone synthase, a dihydroflavonol 4-reductase or a leucoanthocyanine reductase, these are not special technical features because none of these are novel enzymes. For example, the following three journal abstracts indicate that none of these enzymes are novel, and therefore cannot be used as a special technical feature uniting the inventions into three groups.

NAPOLI C, et. al. The Plant Cell (1990) 2:279-89 Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-suppression of Homologous Genes *in trans*.

JOHNSON ET, et. al. Plant Journal (2001) 25(3):325-33 Alteration of a single amino acid changes the substrate specificity of dihydroflavonol 4-reductase.

TANNER GJ et.al. Anal Biochem 1993 March 209(2):274-7 Synthesis of 3,4-cis-[3H]leucocyanidin and enzymatic reduction to catechin

(Continued on Extra Sheet.)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2004/000494

Supplemental Box
(To be used when the space in any of Boxes I to VIII is not sufficient)

Continuation of Box No:

Furthermore, given the nature of the claims and the invention, it is appropriate to apply the Markush approach. Using the Markush approach to analyse the unity of inventions, although the 8 gene transcripts all have the common property (A) that they are involved in flavonoid biosynthesis in plants, and more specifically the modification of the content of condensed tannins, (B)(1) there is no common structure present in all of the genes; and (B)(2) there is no single recognised class or group of compounds embracing all the genes claimed. It is contrary to normal classification to group together such diverse genes. Thus according to Markush, it is appropriate to classify the genes in terms of the 8 individual groups and thus these groups represent 8 different inventions.

In order to search each of the inventions, this could only be done by consideration of each of the sequences, thereby requiring eight separate searches. As a service to the applicant, this office would for the purposes of this search, consider Inventions 1 and 5 together for a single search fee. Each of the other sequences will be considered as single inventions and each attracts a search fee.

The applicant chose to pay the six extra fees being sufficient to search and examine all eight inventions.